JANUARY 1952, VOLUME 50, No. 3

THE BIOCHEMICAL JOURNAL

UNIVERSITY
OF MICHIGAN
FEB 1 1952
MEDICAL
LIBRARY

Edited for

THE BIOCHEMICAL SOCIETY

Editorial Board

R. K. CALLOW
R. E. DAVIES
D. HERBERT
R. A. KEKWICK
E. J. KING (*Chairman*)
W. KLYNE
M. G. MACFARLANE
R. A. MORTON
A. NEUBERGER
A. PIRIE
R. L. M. SYNGE
T. S. WORK

CAMBRIDGE UNIVERSITY PRESS
LONDON: BENTLEY HOUSE, N.W.1
NEW YORK: 32 EAST 57TH STREET, 22

Price 20s. net

The *Biochemical Journal* is conducted by the **Biochemical Society** and is published by the Cambridge University Press. The *Journal* is issued in multiple volumes per year. Each volume consists of approximately 640 pages, published in five parts at intervals of one month.

The Biochemical Society was instituted solely for the advancement of the Science of Biochemistry, and holds meetings at different centres for the communication of original papers, and for the discussion of current problems. Persons interested in Biochemistry are eligible for election. Members whose subscriptions are not in arrears are entitled to receive the *Biochemical Journal* without further payment. Further information may be obtained from the Hon. Secs., Professor J. N. Davidson, Department of Biochemistry, The University, Glasgow, W. 2, and Professor Leslie Young, St Thomas's Hospital Medical School, London, S.E. 1, or from the Hon. Treas., Dr J. H. Bushill, 149 Hammersmith Road, London, W. 14.

Subscriptions to the *Biochemical Journal*. For non-members of the Biochemical Society the subscription to the *Biochemical Journal* is £3. 10*s*. 0*d*. per volume, payable in advance to the Cambridge University Press, Bentley House, 200 Euston Road, London, N.W. 1, to Dr J. H. Bushill, The Laboratories, 149 Hammersmith Road, London, W. 14, or to any bookseller. In the United States of America the *Journal* may be purchased from the Cambridge University Press American Branch, 32 East 57th Street, New York, 22 (subscription price $12.25 per volume).

Communications respecting the sale of single issues or back numbers of the *Journal*, Volumes 17–47, should be addressed to the Cambridge University Press, Bentley House, 200 Euston Road, London, N.W. 1. Volumes 1–15 have been reproduced and are available from Messrs William Dawson & Sons, Ltd., 102 Wigmore Street, London, W. 1, at the prices given below.

Claims for the replacement of *Journals* lost in transmission will not be entertained if they are received later than three months after the date of the posting of the *Journal*.

Prices of back numbers of the *Journal*.

Volumes 1–15 complete.	£82. 10*s*. bound, £75 unbound
Separate volumes.	£6 each bound, £5. 10*s*. each unbound

Reduced terms are available to members of the Biochemical Society, who should apply to the Honorary Secretaries.

In paper covers (except Vol. 38)

Volumes 16–19.	A few odd parts only are available, prices on application
"	The reprinting of these volumes is under consideration
Volumes 20–28.	£8 per volume of 6 parts at £1. 12*s*. each if sold separately
Volumes 29–35.	£8 per volume of 12 parts at 16*s*. each if sold separately
Volume 36.	£5 per volume of 12 parts at 10*s*. each if sold separately
Volumes 37 & 40.	£5 per volume of 6 parts at £1 each if sold separately
Volumes 41–43.	£5 per volume of 4 parts at £1. 10*s*. each if sold separately
Volumes 38, 39, 44–49.	£5 per volume of 5 parts at £1. 4*s*. each if sold separately
Index to Volumes 11–20.	10*s*. net
Index to Volumes 21–30.	15*s*. net
Index to Volumes 31–40.	£1. 15*s*. net
History of the Biochemical Society	2*s*. 6*d*. net

Binding. Quotations can be given by the publishers for bound copies of back numbers; also for buckram binding cases, and for binding subscribers' sets. (Suspended.)

CONTENTS OF VOLUME 50, No. 3, 1952

(*All rights reserved*)

PAGE

The pectic enzymes of the fungus *Byssochlamys fulva*. By W. W. Reid 289

The fermentation process in tea manufacture. 12. The origin of carbon dioxide. By E. A. H. Roberts and D. J. Wood 292

Studies on yeast metabolism. 1. Fractionation and microdetermination of cell carbohydrates. By W. E. Trevelyan and J. S. Harrison. (With two figures) 298

Studies on yeast metabolism. 2. Synthesis of cell carbohydrates during glucose fermentation, and its inhibition by azide. By W. E. Trevelyan, J. N. Gammon, E. H. Wiggins and J. S. Harrison. (With six figures) 303

The preparation and properties of aucubin, asperuloside and some related glycosides. By A. R. Trim and R. Hill. (With two figures) 310

The accumulation and utilization of asperuloside in the Rubiaceae. By A. R. Trim. (With five figures) . 319

A modified medium for *Lactobacillus casei* for the assay of B vitamins. By K. M. Clegg, E. Kodicek and S. P. Mistry. (With two figures) 326

Specificity of glucose oxidase (notatin). By D. Keilin and E. F. Hartree. (With seven figures) . . 331

Biological catalysis of mutarotation of glucose. By D. Keilin and E. F. Hartree. (With four figures) . 341

Estimation of L-malate and fumarate by malic decarboxylase of *Lactobacillus arabinosus*. By P. M. Nossal 349

The composition of the bulk proteins of *Chlorella*. By L. Fowden 355

The branched-chain fatty acids of butterfat. 2. The isolation of a multi-branched C_{20} saturated fatty acid fraction. By R. P. Hansen and F. B. Shorland 358

The oxidation of amines by extracts of pea seedlings. By R. H. Kenten and P. J. G. Mann. (With five figures) 360

Methods of paper chromatography of steroids applicable to the study of steroids in mammalian blood and tissues. By I. E. Bush. (With six figures) 370

Kinetics of 'acid' phosphatase action. By F. Schønheyder. (With five figures) 378

The enzymically catalysed transfer of the deoxyribosyl group from one purine or pyrimidine to another. By W. S. Macnutt. (With six figures) 384

Trans-*N*-glycosidase studied with radioactive adenine. By H. M. Kalckar, W. S. Macnutt and E. Hoff-Jørgensen 397

A microbiological assay of deoxyribonucleosides and deoxyribonucleic acid. By E. Hoff-Jørgensen. (With one figure) 400

The enzymic oxidation and assay of adenine. By H. Klenow. (With three figures) 404

Studies involving enzymic phosphorylation. 1. The hexokinase activity of rat tissues. By C. Long . . 407

Blood clotting: the function of electrolytes and of calcium. By J. E. Lovelock and B. M. Porterfield. (With two figures) 415

Enzyme inhibition by suramin and the measurement of the isoelectric points of some enzymes. By E. D. Wills. (With four figures) 421

Astaxanthin, cholesterol and lipins in developing salmon eggs. By M. Glover, R. A. Morton and D. G. Rosen 425

Some observations on the zinc in carbonic anhydrase. By R. Tupper, R. W. E. Watts and A. Wormall . 429

Proceedings of the Biochemical Society

PAGE

Colorimetric determination of the β-glucuronidase activity of locust crop fluid using 8-glucuronosidoquinoline as substrate. By D. Robinson, J. N. Smith and R. T. Williams xiii

Biochemistry of fluoroacetate poisoning. Isolation of a crystalline inhibitor of citrate metabolism. By R. A. Peters, R. W. Wakelin and P. Buffa xiii

Direct spectrochemical micro-estimation of organically bound fluorine using the hollow-cathode. By A. H. C. P. Gillieson and R. A. Newcombe xiv

Naturally occurring L-pipecolinic acid. By R. I. Morrison xiv

The inhibition of β-glucuronidase by derivatives of saccharic acid. By G. A. Levvy xv

Some applications of a new method for the synthesis of hexuronides. By G. A. Levvy, C. A. Marsh and P. G. Walker xvi

Phospholipin metabolism in the lactating mammary gland of the rabbit. By G. A. Garton and G. Popják xvi

Non-available water in precipitates formed by antigens and antibodies. By J. R. Marrack, R. A. Grant and R. G. S. Johns xvii

The effect of dioxan on the formation of precipitates by antigens and antibodies. By R. A. Grant . . xvii

Estimation of glucosamine in the presence of protein. By R. G. S. Johns and J. R. Marrack . . . xvii

Transfructosidation by a yeast invertase preparation. By J. S. D. Bacon xviii

Bicarbonate ion and muscle potassium. By R. Creese xviii

Chemistry of connective tissue fibrinoid in rheumatic fever. By R. Consden, L. E. Glynn and W. M. Stanier xix

Thiocyanates in milk and other biological fluids. By F. Wokes, P. Wedgwood and J. Wyatt . . xix

I. Acetoin and 2:3-butylene glycol formation by washed cells of *Aerobacter aerogenes*. II. The enzymic formation of acetoin by preparations from (*a*) *Aerobacter aerogenes*, (*b*) Animal tissues. By C. P. Spencer, F. C. Happold, R. P. Hullin and R. L. Noble xx

The formation of acetoin and 2:3-butylene glycol in the decerebrate cat. By J. Dawson and R. P. Hullin xx

The enzymic phosphorylation of D-glucosamine. By P. T. Grant and C. Long xx

The biosynthesis of L-cystine and L-methionine labelled with ^{35}S. By R. B. Williams and R. M. C. Dawson xxi

The detection of chromatogram spots by means of ultraviolet radiation. By G. H. Beaven, E. R. Holiday and E. A. Johnson xxi

A simple automatic fraction collector for preparative chromatography. By J. Edelman and R. V. Martin xxi

INDEX OF AUTHORS

	PAGE
Bacon, J. S. D.	xviii
Beaven, G. H.	xxi
Buffa, P.	xiii
Bush, I. E.	370
Clegg, K. M.	326
Consden, R.	xix
Creese, R.	xviii
Dawson, J.	xx
Dawson, R. M. C.	xxi
Edelman, J.	xxi
Fowden, L.	355
Gammon, J. N.	303
Garton, G. A.	xvi
Gillieson, A. H. C. P.	xiv
Glover, M.	425
Glynn, L. E.	xix
Grant, P. T.	xx
Grant, R. A.	xvii
Hansen, R. P.	358
Happold, F. C.	xx
Harrison, J. S.	298, 303
Hartree, E. F.	331, 341
Hill, R.	310
Hoff-Jørgensen, E.	397, 400
Holiday, E. R.	xxi
Hullin, R. P.	xx
Johns, R. G. S.	xvii
Johnson, E. A.	xxi
Kalckar, H. M.	397
Keilin, D.	331, 341
Kenten, R. H.	360
Klenow, H.	404
Kodicek, E.	326
Levvy, G. A.	xv, xvi
Long, C.	407, xx
Lovelock, J. E.	415
Macnutt, W. S.	384, 397
Mann, P. J. G.	360
Marrack, J. R.	xvii
Marsh, C. A.	xvi
Martin, R. V.	xxi
Mistry, S. P.	326
Morrison, R. I.	xiv
Morton, R. A.	425
Newcombe, R. A.	xiv
Noble, R. L.	xx
Nossal, P. M.	349
Peters, R. A.	xiii
Popják, G.	xvi
Porterfield, B. M.	415
Reid, W. W.	289
Roberts, E. A. H.	292
Robinson, D.	xiii
Rosen, D. G.	425
Schønheyder, F.	378
Shorland, F. B.	358
Smith, J. N.	xiii
Spencer, C. P.	xx
Stanier, W. M.	xix
Trevelyan, W. E.	298, 303
Trim, A. R.	310, 319
Tupper, R.	429
Wakelin, R. W.	xiii
Walker, P. G.	xvi
Watts, R. W. E.	429
Wedgwood, P.	xix
Wiggins, E. H.	303
Williams, R. B.	xxi
Williams, R. T.	xiii
Wills, E. D.	421
Wokes, F.	xix
Wood, D. J.	292
Wormall, A.	429
Wyatt, J.	xix

The Pectic Enzymes of the Fungus *Byssochlamys fulva*

By W. W. REID
Research Department, H. W. Carter and Co. Ltd., Coleford, Gloucestershire

(*Received* 17 *April* 1951)

It was reported by Beavan & Brown (1949) that cultures of *Byssochlamys fulva* Olliver & Smith produced protopectinase, but neither polygalacturonase nor pectin esterase; they concluded that *B. fulva* produced a disaggregating enzyme, which lowered the viscosity of pectin solutions, without the formation of free reducing groups. A re-investigation of the problem has been made, and in a preliminary note (Reid, 1950*a*), it was suggested that *B. fulva* produced both polygalacturonase and pectin esterase.

EXPERIMENTAL

Enzyme preparations

Two strains of *Byssochlamys fulva* were used; one was received from Mr L. D. Galloway (I) and the other from Miss M. Olliver (II). *B. fulva* is considered by Thom & Raper (1945) to be an ascoporic phase of *Paecilomyces varioti* and isolates of the non-ascoporic phase have been found to produce small amounts of pectic enzymes (unpublished data).

Previous work with other microfungi had shown that several cultures produced good yields of enzymes on bran. In some fungi, e.g. *P. chrysogenum* (Phaff, 1947), the enzyme was adaptive, in others (e.g. *Aspergillus foetidus* Thom & Raper), the nitrogen source was all important. In the present work, cultures were grown on bran supplemented with organic nitrogen, e.g. peptone or protein hydrolysates. When the enzyme formation was optimal, the culture was extracted with chloroform water and the extract was either dialysed against running tap water, followed by distilled water, or precipitated with acetone (70% v/v) at 4° and vacuum dried.

Estimation of enzyme activity

Viscometric method. A 'four-point' viscometric assay was used (Reid, 1949; Ayres, Hooper & Reid, 1951) with sodium pectate at pH 5·0 for polygalacturonase and citrus pectin at pH 3·5 as an approximate estimate of combined pectin esterase and polygalacturonase activity. A preparation of Pectinol 10 *M* (Rohm and Haas Co., Philadelphia) was preserved over silica gel at 4° as a standard.

Glycosidic hydrolysis. (*a*) The enzyme was added to a 1% solution of sodium pectate at pH 5·0 and 25°. Samples were withdrawn at intervals and titrated by the hypoiodite method of Willstätter & Schüdel (cf. Hinton, 1939) against D-galacturonic acid as a standard.

(*b*) The colorimetric method of Sumner (1924–5), as modified by Smith & Stocker (1949), was used. The sample to be analysed was brought to a total volume of 5 ml., 1 ml. of the dinitrosalicylic acid reagent added, and the tubes heated for 15 min. in a boiling-water bath and cooled rapidly in running water. The red-brown colour which developed was read in a Hilger Biochem Absorptiometer with a green OGI filter. A standard curve was constructed using D-galacturonic acid, and the linear portion (0·7–2·0 mg. polygalacturonic acid) was used.

Pectin esterase assay. (*a*) The titrimetric method of Kertesz (1937) was used, and the enzyme solution was well dialysed to remove buffering substances. Methyl red was used as an indicator, the solution was made 0·1 M with NaCl, and 0·2 N-NaOH added continuously from a microburette to maintain the pH at 6·0.

(*b*) The enzyme solution was added to a 2% solution of citrus pectin at pH 6·0. Samples were removed and distilled at intervals and the methanol in the distillate estimated by the Denigès method (modified by the A.O.A.C., 1945). Methanol (British Drug Houses Ltd., Analar grade) was used as a standard.

Substrates

Pectin. 100 grade pectin (British Drug Houses Ltd.) was well washed with 70% (v/v) ethanol to remove soluble carbohydrate, then with absolute ethanol and dried *in vacuo*. For the Kertesz (1937) method for estimating pectin-esterase activity, the buffer substances present were removed, either by acidifying a 1% solution with HCl followed by dialysis against distilled water or by shaking a 1% solution with Bio-Deminrolit (Permutit Co. Ltd.).

Pectic acid. To a 1% solution of 100 grade pectin, NaOH solution (33%, w/v) was added to pH 10·0 while stirring in a Waring blender. After 1 hr. the solution was adjusted to pH 1·0 with HCl (10%, v/v). The pectic acid gel was pressed in a small screw press, washed with 70% ethanol containing 1% HCl followed by 70% ethanol until free from HCl, left under absolute ethanol for 24 hr., the excess ethanol pressed out, and the fibrous mass dried *in vacuo* at 50–70° for several hours.

The substrates were dispersed to the appropriate concentrations in either distilled water or buffer (McIlvaine) in the cold.

RESULTS

In a preliminary investigation it was shown (Table 1) that, compared with other cultures, *Byssochlamys fulva* (II) was active in the production of pectic enzymes, whereas (I) was very poor. The 'high level' enzyme-substrate mixtures from the assay of the two *B. fulva* cultures and the Pectinol standard were maintained at 25° for 18 hr. Sodium hydroxide was added to pH 10·0, the mixture left for 30 min. to

demethylate the uronides present, and a calcium pectate determination carried out (cf. Hinton, 1939). From the weight and appearance of the resulting calcium pectate precipitates (Table 2) the enzyme action had markedly changed the colloidal properties of the pectin. A similar result was obtained by precipitating reaction mixtures with acetone (70%, v/v); in one such experiment the resulting precipitate from the original substrate was 370 mg., whereas two other samples treated for 20 hr. with different extracts of *B. fulva* (II) gave precipitates of 221 and 150 mg.

Table 1. *The pectic enzyme activity of 5-day cultures (dry basis) of microfungi, by a viscometric 'four point' assay on* 0·4% *pectin at* pH 3·5

(Activity of culture compared with that of Pectinol 10*M* used as standard *M* indicator, arbitrary enzyme unit.)

Organisms	Temp. of incubation (°)	Assay value
P. chrysogenum	25	1·2 *M*
Stachybotrys spp.	25	1·2 *M*
Monilia spp.	25	0·1 *M*
Aspergillus foetidus, n.sp. Thom & Raper	37	3·0 *M*
B. fulva (I)	37	0·04 *M*
B. fulva (II)	25	0·9 *M*
B. fulva (II)	37	0·7 *M*

The polygalacturonase activity of Byssochlamys fulva

The marked activity of the *B. fulva* preparations suggested that polygalacturonase was present, and preliminary experiments indicated the formation of reducing material during enzyme action. In a later experiment, 50 ml. of culture extracts of *B. fulva* (II) were precipitated with acetone at 4°. The white precipitate obtained was redissolved, centrifuged from a trace of residue and the clear extract (5 ml.) added to 50 ml. of a 1% solution of a pectic acid in McIlvaine buffer at pH 5·0. The reducing value after 48 hr. (hypoiodite method) corresponded to a D-galacturonic acid content of 63·3–65·5% of the theoretical. Paper chromatography from this experiment showed no soluble sugars in the enzyme and substrate blanks, but D-galacturonic acid and some higher uronides in the enzyme substrate mixtures. These results confirmed that polygalacturonase was present (see also Table 3).

Examination of the activity of several extracts from *B. fulva* (I) and (II) for polygalacturonase by the 'cup-plate' assay, Reid (1950*b*) showed that small but definite amounts of the enzyme were present, as was also the case with approximately a hundred other cultures of microfungi from different genera, indicating that polygalacturonase formation is a common feature among microfungi (Solomons, 1951).

Table 2. *The action of* Byssochlamys fulva *extracts and Pectinol* 10*M on* 0·4% *pectin at* pH 3·5 *for* 18 *hr. as measured by the calcium pectate value*

	Character of calcium pectate ppt.	Calcium pectate (mg./20 ml.)	Calcium pectate remaining (%)
Pectin substrate	Gel-like	80·1	100·0
Pectin substrate + *B. fulva* (I)	Gel-like	79·0	98·6
Pectin substrate + *B. fulva* (II)	Flocculent ppt.	69·5	86·8
Pectin substrate + Pectinol 10*M*	Flocculent ppt.	70·2	86·2

Table 3. *Reducing substances formed and methanol liberated by the action of* Byssochlamys fulva *preparations and Pectinol* 10*M, on pectin at* pH 5·0 *for* 120 *hr.*

(Reducing substances determined by the hypoiodite method on 2 ml. enzyme-substrate mixture, and methanol determined in the distillate from 2 ml. enzyme-substrate mixture, nos. 1–4, 50 ml. 2% pectin (9·0% MeO) + enzyme + water to 55 ml.)

Exp. no.	Additions	Reducing material formed (as mg. D-galacturonic acid/2 ml.)	Methanol liberated (mg./2 ml.)	Methanol liberated (% total methoxyl)
1	4 ml. Pectinol + 1 ml. water	22·23	2·2	61·1
2	As 1, but enzyme heat-inactivated	0·0	0·0	—
3	5 ml. solution of acetone-precipitated *B. fulva* preparation	7·98	2·0	55·5
4	As 3, but enzyme heat-inactivated	0·0	0·0	—
5	50 ml. 2 pectin in pH 5·0 buffer + 50 ml. dialysed *B. fulva* extract	12·54	1·1	61·1
6	As 5, but enzyme heat-inactivated	0·0	0·0	—

The relationship between reducing groups liberated and reduction in viscosity with a pectic substrate

As polygalacturonase was present in the enzyme preparations, it seemed likely that the failure of Beavan & Brown (1949) to detect any liberation of reducing groups was due to the very low activity of their enzyme preparations. Examination of their data indicated that to reduce the specific viscosity $\eta_{sp.}^{25}$ of a substrate to 50 % of the initial value, a reaction time of approximately 12 hr. was necessary, whereas the majority of the preparations in the present paper required 20–25 min.

Preparations of *B. fulva* (II) were compared with Pectinol 10*M*, the latter being taken as typical of a preparation of high polygalacturonase content. An attempt was made to examine the liberation of reducing groups using amounts of each preparation that would give a rate of reduction in viscosity comparable to that given in Fig. 1 (in Beavan & Brown, 1949). The amount of reducing material was too small to be detected by the hypoiodite method, but just detectable by the colorimetric method of Sumner (1924–5). The latter method was more sensitive than the hypoiodite method, and gave good recoveries for various dilutions of pectin, and pectin-enzyme solutions. Although it was possible to demonstrate small amounts of reducing material by the colorimetric method in the early stages of enzyme action, the results were subject to considerable error. However, by taking larger amounts of *B. fulva* preparations and Pectinol, estimating the glycosidic hydrolysis, and extrapolating the curve back to the zero time, it was found that for a 50 % drop in viscosity the accompanying glycosidic hydrolysis was 0·5–1·0 % of the theoretical.

Pectin-esterase activity of Byssochlamys fulva

Using the Kertesz (1937) titrimetric method it was found essential to free both substrates and enzyme solutions from buffer substances. In typical experiments 40 ml. of dialysed culture extracts were added to 40 ml. 4 % pectin solution containing methyl red and sodium chloride to 0·2 M. The pH of the mixture was adjusted to 6·0 with 0·2 N-sodium hydroxide, and with the majority of the preparations there was marked acid production in 1–2 hr. at 25°. With concentrated enzyme preparations prepared by dialysis and solvent precipitation, the acid produced could be titrated continuously. One such preparation produced acid at an initial rate equal to 2·4 ml. 0·2 N-sodium hydroxide/hr. In the nomenclature of Kertesz (1937) this was equivalent to 0·093 P.M.U. (pectin methyl esterase units) in 0·1 M-sodium chloride, at pH 6·0 and 25° per ml. of enzyme solution.

To confirm that the acid production was in fact due to demethylation of the pectin, samples of a *B. fulva* preparation and heated control samples were added to a pectin solution at pH 6·0, and samples distilled at intervals and the methanol in the distillate determined. From Table 3 it may be seen that methanol was liberated during the enzyme action.

Arabanase and galactanase of Byssochlamys fulva

An enzyme preparation was added to a solution of apple pectic acid, buffered to pH 5·0, which had been freed from soluble sugars, but contained considerable araban and galactan. Paper chromatograms after 24 hr. reaction showed no soluble sugars in the enzyme and substrate solutions alone, but with the enzyme-substrate mixture, in addition to uronides, well defined spots of arabinose and galactose were obtained, indicating arabanase and galactanase in the enzyme solution.

DISCUSSION

It is clear from the above results that *B. fulva* produces the pectin-esterase, polygalacturonase, arabanase and galactanase necessary to degrade the pectin complex. The action of these enzymes in pectin results in a drop in viscosity and the formation of reducing groups, but for reasons previously discussed (Jermyn & Tompkins, 1950; Reid, 1950*c*), the exact relationship between these two effects is difficult to assess. In the present work, a 50 % drop in viscosity was accompanied by a glycosidic hydrolysis of 0·5–1·0 % theoretical. As the preparations of Beavan & Brown reduced the viscosity of pectin slowly, it follows that the amount of glycosidic hydrolysis was very small; for example in Table 2 of Beavan & Brown's paper an increase in reducing value during enzyme action did occur, equivalent to 0·02–0·05 ml. 0·1 N-iodine, which may in fact have been a real increase, although it was of the same magnitude as the error of the determination. It would appear that Beavan & Brown were unable to detect pectin-esterase and polygalacturonase activity due to the low activity of their preparations; this low activity may have been in part due to the fact that they grew their cultures on Czapek solution, a medium which often produces weak extracts, and precipitated the extracts with ethanol at room temperature, a procedure which markedly inactivates pectic enzyme preparations.

In the light of these findings there is no evidence for the presence in *B. fulva* of a special 'disaggregating enzyme' as postulated by Beavan & Brown, although as suggested previously Reid (1950*c*) arabanase and galactanase may contribute to the degradation brought about by polygalacturonase.

SUMMARY

1. It has been shown, contrary to the results of Beavan & Brown (1949), that *Byssochlamys fulva* produces pectin esterase and polygalacturonase.

2. It is suggested that the results of Beavan & Brown were due to the low activity of their enzyme preparations, which made the detection of pectin esterase and polygalacturonase very difficult by the methods they used.

Thanks are due to Miss M. Olliver and Mr L. D. Galloway for cultures of *B. fulva*, to Mr G. L. Solomons and Mr A. Phipps for experimental assistance and the Directors of H. W. Carter and Co. Ltd., for permission to publish this paper.

REFERENCES

A.O.A.C. (1945). *Methods of Analysis of the Association of Official Agricultural Chemists*, 6th ed. p. 717. Washington, D.C.

Ayres, A., Hooper, F. & Reid, W. W. (1951). Paper in preparation.

Beavan, G. H. & Brown, F. (1949). *Biochem. J.* **45**, 221–4.

Hinton, C. L. (1939). Fruit pectins. Their Chemical Behaviour and Jellying Properties. *Spec. Rep. Food Invest. Bd, Lond.*, no. 48. H.M.S.O.

Jermyn, M. A. & Tompkins, R. G. (1950). *Biochem. J.* **47**, 437.

Kertesz, Z. I. (1937). *J. biol. Chem.* **121**, 589.

Phaff, H. S. (1947). *Arch. Biochem.* **13**, 67.

Reid, W. W. (1949). *1st Int. Congr. Biochem. Abstr.* p. 287.

Reid, W. W. (1950*a*). *Nature, Lond.*, **166**, 76.

Reid, W. W. (1950*b*). *Nature, Lond.*, **166**, 569.

Reid, W. W. (1950*c*). *J. Sci. Food Agric.* **1**, 234.

Smith, G. N. & Stocker, C. (1949). *Arch. Biochem.* **21**, 95.

Solomons, G. L. (1951). Unpublished observations.

Sumner, J. B. (1924–5). *J. biol. Chem.* **62**, 289.

Thom, C. & Raper, K. B. (1945). *A Manual of the Aspergilli*, p. 219. London: Baillière, Tindall and Cox.

The Fermentation Process in Tea Manufacture

12. THE ORIGIN OF CARBON DIOXIDE

By E. A. H. ROBERTS and D. J. WOOD

Tocklai Experimental Station, Indian Tea Association, Cinnamara, Assam, India

(*Received* 3 *March* 1951)

There are considerable differences of opinion on record regarding the origin of the carbon dioxide produced in fermentation, and the extent to which rolling and fine mincing destroy respiratory activity in the tea leaf.

Sreerangachar (1941, 1949) is of the opinion that there is much virtually undamaged tissue both in the fine minces used in manometric work, and in factory-rolled leaf, and that the carbon dioxide evolved during fermentation arises from normal respiration in such undamaged leaf. He produced experimental evidence which showed that the minces used in his manometric experiments contained appreciable amounts of intact tissue, but, as will be shown below, the same criteria when applied to our minces indicate a much greater degree of homogenization.

Roberts (1941*c*), on the other hand, concluded that fine mincing results in almost complete suppression of respiratory activity. This was indicated by the absence of anaerobic fermentation in a fine mince (Deb & Roberts, 1940), and by the low rates of oxygen uptake and carbon dioxide output observed in minces after polyphenol oxidation was complete (Roberts, 1939). It was pointed out that carbon dioxide output in a mince runs parallel with oxygen uptake and that ascorbic acid, which reduces *o*-quinones, completely stops carbon dioxide output (Roberts, 1939). This output was believed, therefore, to depend upon oxidation of the polyphenols.

In the fermentation of factory-rolled leaf the Löwenthal non-tan titre was observed to decrease (Roberts & Sarma, 1938). The non-tans were then thought to include various respiratory substrates. A fall in reducing sugar content on fermentation was also recorded (Roberts, 1939) and it was suggested that reducing sugars and other respiratory substrates in the non-tan fraction were oxidized to carbon dioxide by the *o*-quinones produced in fermentation, the *o*-quinones functioning as H-acceptors in the dehydrogenations involved. Depletion of carbohydrates in the tea leaf by water culture in the dark for 3 days would therefore be expected to reduce carbon dioxide output in the fermentation of a leaf mince. As Sreerangachar (1941) has pointed out, the failure of Deb & Roberts (1940) to observe such an effect is inconsistent with the view that carbon dioxide is formed as a result of the oxidation by *o*-quinones of glucose and other respiratory substrates.

Roberts & Wood (1950) have shown that the polyphenols in the tea leaf yield considerable amounts of carbon dioxide on enzymic oxidation. This explains the parallelism of oxygen uptakes with carbon dioxide outputs, and also accounts satisfactorily for the amount of carbon dioxide produced, without having to postulate oxidation of substances such as glucose.

In this communication we present further evidence which establishes the almost complete absence of normally respiring tissue in our leaf minces, and the considerable inactivation of respiratory activity which occurs during tea manufacture as a result of the rolling process. The possible origin of carbon dioxide as a product of oxidation of glucose and other oxidizable substances in the leaf is considered, and shown to be extremely unlikely in a mince and of not more than secondary importance in the manufacturing process.

METHODS AND MATERIALS

Mincing of tea leaf samples. Tea leaf is minced in a domestic mincing machine using an attachment designed for grinding nuts to a paste. The fragments consist of two distinct categories. The smaller, numerically preponderant particles range from 100 μ. downwards, and consist almost entirely of cell debris, pieces of broken cell wall, and occasional chloroplasts. Most of these particles exhibit Brownian movement. The larger particles, which account for the greater proportion of the mass of the mince, range in size from $170 \times 90\,\mu$. up to $970 \times 750\,\mu$., averaging $500 \times 350\,\mu$.

Manometric methods. For minces both the direct method of Warburg and the first method of Dickens and Šimer were employed (Dixon, 1934). For leaf disks the technique was as described by Deb & Roberts (1940). The extent of polyphenol oxidation in fermented leaf was determined by the residual uptake method (Roberts, 1941*b*, 1949), the total uptake recorded in 2 hr. giving an estimate of the polyphenols still unoxidized.

Enzyme preparations and substrates. These are as described by Roberts & Wood (1950).

Estimations of lipids. This procedure, which replaces methods involving extraction of the dried product in a Soxhlet with ether or light petroleum, is a combination of the methods described by Nielson & Bohart (1944) and Reichert (1944).

Finely minced leaf (10–20 g.) is refluxed for 15 min. with ethanol (50–100 ml.). After filtration the residue is washed twice with hot ethanol and the washings combined with the filtrate. The residue is dried and refluxed for 10 min. with light petroleum (60–80°) with which the tissue is allowed to remain in contact overnight. The solvent is removed by filtration and the residue washed with light petroleum. The washings are combined with the filtrate.

The residue from the light petroleum extraction is again refluxed with ethanol (25–50 ml.), filtered, washed with ethanol, and the extract and washings combined. The two ethanol extracts are mixed, NaCl (1 g.) added, and the mixture concentrated to a small volume, consisting essentially of an aqueous solution of ethanol-soluble matter with a layer of fats swimming on the surface. Subsequent treatment with light petroleum and estimation of total lipid is as described by Nielson & Bohart (1944).

Estimation of carbon dioxide evolved from factory-treated leaf. This was estimated by a Pettenkofer method as described by Deb & Roberts (1940).

RESULTS

Determination of respiratory activity in fine minces

Washing of minced leaf. The effect of washing a fine mince of tea leaf is to reduce both oxygen uptake and carbon dioxide output, and the R.Q. is also appreciably diminished (Table 1). This contrasts strongly with the results of Sreerangachar (1941), and suggests that our mince, unlike that of Sreerangachar's, contains negligible proportions of intact, normally respiring tissue.

Table 1. *Effect of washing a fine mince*

(Minced tea leaf (2 g.) was washed twice with water (30 ml.) and respiratory activity determined on samples equivalent to 200 mg. of the original mince.)

	After 2 hr. shaking		
	(μl. O_2)	(μl. CO_2)	R.Q.
Original mince	460	175	0·38
Washed mince	47	10	0·21

Effect of glucose addition on fermentation. Addition of glucose to a leaf mince has no significant effect on the oxygen consumed in fermentation (Table 2). This is again in contrast with Sreerangachar's (1941) findings.

Table 2. *Effect of addition of glucose on fermentation*

(Minced tea leaf, 150 mg., suspended in water. Total volume 3 ml.)

Glucose concn. (M) ...	0·0	0·01	0·05	0·50
Time (min.)	O_2 uptake (μl.)			
10	108	106	111	105
30	204	198	204	204
60	231	225	228	229
90	237	230	239	234

Polyphenol oxidation in a fine mince. Oxygen uptakes were measured for systems consisting of tea leaf mince suspended in water, and of a 1 hr. infusion of the same mince in water, together with washed enzyme. The uptake with a 1 hr. infusion is about 77 % of that realized with the mince. Independent checks show that about 80 % of the water-soluble matter is extracted in 1 hr. when mince is infused with water under these conditions.

Fermentation in factory-rolled leaf

In north-east India it is customary to give the withered leaf two successive rolls of about 30 min. duration each. Table 3 shows residual oxygen uptakes determined for samples of leaf from a commercial factory, which had received one and two 30 min. rolls respectively. The second roll has resulted in very little extra fermentation.

Table 3. *Residual uptakes of fermented tea*

(Rolled leaf allowed to ferment for 3 hr. and then finely minced. 150 mg. portions suspended in 3 ml. water.)

Date of manufacture	Residual uptake in 2 hr. (μl. O_2/mg. dry wt.) No. of rolls 1	2
19. v. 49	2·84	2·27
31. v. 49	3·22	2·86
16. vi. 49	1·98	1·49
23. vi. 49	1·01	1·02

Polyphenol oxidation is still incomplete after a 3 hr. fermentation in the factory, and given sufficient time, further oxygen will be consumed. Judging from figures available for residual uptakes after different lengths of fermentation (Roberts, 1949) and for total uptakes for green leaf (Roberts, 1941*c*), 90 % or more oxidation of the polyphenols will result eventually after one roll.

Carbon dioxide production in leaf exposed to chloroform vapour and in leaf warmed to 55°

When tea leaf is exposed to chloroform vapour, or maintained at a temperature of 50–55° (Roberts, 1941*a*) the polyphenols undergo irreversible oxidation and condensation in the same way as in fermentation, and the whole leaf assumes a uniform brown colour. A manometric study has been made of the respiratory activity of leaf disks subjected to such treatments (Table 4), and it is found that the disks behave in much the same way as a fine mince. There is a period of rapid oxygen uptake followed by a smooth die-away with the carbon dioxide output running approximately parallel with the oxygen uptake throughout the whole period of the experiment.

Table 4. *Effects of chloroform vapour and of heating on respiratory activity of green leaf*

(Exp. 1, leaf disks (250 mg.) exposed to $CHCl_3$ vapour for 20 min. Exp. 2 leaf disks (200 mg.) in 3 ml. water maintained at 50–55° for 15 min.)

Time (min.)	Exp. 1 (μl. O_2)	Exp. 1 (μl. CO_2)	Exp. 2 (μl. O_2)	Exp. 2 (μl. CO_2)
15	38	18	66	12
30	71	36	124	20
60	135	66	207	37
120	200	100	323	60
180	237	118	403	76
240	256	129	—	—

Total uptake after chloroform treatment is lower than after heat treatment. This is probably due to a quicker initiation of fermentation by chloroform so that appreciable fermentation has already taken place by the time that the chloroform-treated disks of leaf have been weighed out into the manometric vessels. This would obviously decrease the total uptake measured.

Association of carbon dioxide output with dehydrogenase activity

Effect of inhibitors. The effect of arsenite on the respiration of tea leaf is a significant depression of both oxygen uptake and carbon dioxide output, but this inhibitor is without significant effect upon the fermentation of a fine mince from the same bulk of leaf (Table 5). Iodoacetate (0·05 M), malonate (0·05 M) and 1-naphthol-2-sulphonic acid (0·01 M) are all without significant effect on either oxygen uptake or carbon dioxide output of a leaf mince.

Table 5. *Effect of arsenite on respiration and fermentation*

(Fermentation 150 mg. mince; respiration, 300 mg. leaf disks.)

	After 1 hr. shaking: Fermentation (μl. O_2)	Fermentation (μl. CO_2)	Respiration (μl. O_2)	Respiration (μl. CO_2)
Control	312	108	207	228
+0·01 M-arsenite	302	95	111	144

Effect of cozymase. Addition of cozymase is without significant effect upon oxygen uptake or carbon dioxide output of minced tea leaf.

Table 6. *Effect of partial heat inactivation on* R.Q.

(Leaf fermented 3 hr. at 37°, finely minced and suspended in 3 ml. green leaf infusion, 20 g./200 ml.)

Time (min.)	(μl. O_2)	(μl. CO_2)
30	171	49
60	296	99
90	354	126
120	384	140
180	417	157

Differential inactivation by heat. Results presented in Table 6 show that when fresh green leaf infusion is added to a fine mince of previously fermented leaf, the R.Q. is 0·38 which is the same as for a mince of fresh green leaf.

Relation of R.Q. *to complexity of system.* Table 7 records oxygen uptakes and carbon dioxide outputs for systems consisting of (1) minced leaf, (2) washed enzyme and green leaf infusion, and (3) washed enzyme and a tea catechin preparation. No significant difference in the R.Q.'s is apparent, although the two latter systems are unlikely to contain the factors necessary to enable *o*-quinones to oxidize substances such as glucose.

Table 7. R.Q.'s *of leaf mince and of enzyme and substrate systems*

(O_2 uptakes and CO_2 outputs determined by method of Dickens & Šimer.)

	After 70 min.		
	(μl. O_2)	(μl. CO_2)	R.Q.
Leaf mince, 75 mg.	118	44	0·37
Washed enzyme, 40 mg. +green leaf infusion, 2 ml.	156	60	0·38
Washed enzyme, 40 mg. +tea catechins, 10 mg.	157	59	0·38

Oxidation of substances, other than polyphenols by o-quinones

Table 8 shows the amounts of carbon dioxide evolved when an infusion of green tea leaf, or an infusion from which polyphenols have been removed, is added to a mixture of tea oxidase and catechol. Refluxing an infusion with acid converts the catechins into reddish-brown insoluble products,

Table 8. *Formation of carbon dioxide from non-polyphenols*

(All systems contain washed enzyme, 40 mg. and catechol, 15 mg. at pH 5·6. 1 ml. of the solution under test is added in each case: Dickens & Šimer method.)

Solution added	(μl. CO_2)	Time of shaking (min.)
Nil	5	50
Green leaf infusion (20 g./200 ml.)	127	50
Same after hydrolysis with 2·5 N-HCl and extraction with ether	11	50
Same after continuous extraction with ethyl acetate	29	70
Hibiscus infusion (20 g./100 ml.)	11	80

but also produces free gallic acid as a result of the hydrolysis of galloyl esters. This gallic acid is removed on continuous extraction with ether, and the resulting solution then yields comparatively little carbon dioxide when mixed with oxidase and catechol. Continuous extraction of the green leaf infusion with ethyl acetate removes all catechins, but not the polyphenolic acid believed to be *m*-digallic acid (Roberts & Wood, 1951*a*), and the effect of this removal of catechins is a substantial reduction in the output of carbon dioxide when the extracted infusion is treated with oxidase and catechol. The table also shows a low carbon dioxide output for an infusion of *Hibiscus rosa-sinensis* leaf. This was shown by Roberts & Sarma (1940) to be of low polyphenol content.

There is no significant effect on total uptake when glucose, glucose-1-phosphate, caffeine, malic acid, citric acid, glycine, aspartic acid, glutamic acid and arginine are added either to a green leaf mince or to systems of washed enzyme and (+)-catechin buffered at pH 5·6. Data for glucose are given in Table 2, and the effects of additions of the other substances are essentially similar. None of these substances therefore appears to be oxidized by the *o*-quinones produced during tea fermentation.

In leaf fermented in the factory, fermentation appears to have no appreciable effect on the intensities of the amino-acid spots on two-way paper chromatograms (unpublished observations) so that appreciable oxidation of amino-acids during fermentation appears unlikely.

Previous estimations of ether-soluble matter (Roberts, 1942) indicated a relatively low lipid content (2%) in tea leaf, which was considerably reduced apparently as a result of fermentation. Using a revised procedure the lipid content is now found to be about 8%, but no significant reduction is observed as a result of fermentation.

Estimation of carbon dioxide output from factory-rolled leaf

Carbon dioxide evolution by rolled leaf continues for 5 hr. or more (Table 9), and this evolution is obviously not parallel with the oxygen uptake. The

Table 9. *Evolution of carbon dioxide by fermenting leaf*

(CO_2 evolution was determined on 100 g. samples of rolled leaf. Retention was estimated on 15 g. samples. Residual uptakes were determined with 150 gm. samples of a fine mince of the rolled leaf.)

Time (hr.)	CO_2 output (μl./mg. dry wt.)	Residual uptake (μl. O_2/mg. dry wt.)	CO_2 retention (μl./mg. dry wt.)
0	—	5·13	1·42
1	0·305	—	—
2	0·752	1·54	2·22
3	1·130	—	—
4	1·370	1·10	—
5	1·528	—	—

average R.Q. for the first 2 hr. after rolling may be calculated from these results to be 0·21 and for the next 2 hr., 1·40. This latter figure suggests that some of the carbon dioxide initially produced is temporarily retained by the tissue, to be released slowly over a period of hours. Retention of carbon dioxide, as determined by the method of Wardlaw & Leonard (1939), is considerable and is also affected

by fermentation. This variable retention complicates estimations of carbon dioxide output and a proper study of this has yet to be made.

DISCUSSION

Respiratory activity in a fine mince

According to Sreerangachar (1941) even the finest mincing is unlikely to result in the disruption of every cell, so that some normal respiratory activity is likely to persist. It is not our belief, however, that such disruption, either in a mince or in factory-rolled leaf, is necessary to bring about fermentation. Rupture of the vacuolar membrane or an increase in its permeability to polyphenols is probably sufficient to initiate fermentation, and such damage may leave the outer cell walls intact. In leaf which has lost its turgor, by withering to 70% moisture or less, we consider the twisting which can be imparted during rolling is sufficient to produce the necessary shearing stress required to rupture these membranes. In the fine mince the leaf has also been subjected to considerable shearing stresses, and it appears probable to us that the effects of this shear will not be restricted to cells actually cut, but will extend over a distance of several cell diameters. Few of the larger particles in our mince have a length greater than that corresponding with twenty cell diameters, and rupture of vacuolar membranes in practically all cells in particles of this size appears by no means unlikely.

There is little doubt that respiratory activity in our minces is negligible. The minces obtained by Sreerangachar (1941), however, are probably coarser and contain appreciable proportions of intact, normally respiring tissue. This essential difference is indicated by the different results obtained by Sreerangachar (1941) and ourselves, on washing a mince, and on adding glucose to a mince. The absence of normally respiring tissue from our minces is also indicated by the comparison between oxygen uptakes of the mince suspended in water, and of a mixture of washed enzyme and an infusion of the same mince. Allowing for the incomplete extraction of water-soluble matter when the mince is infused for 1 hr., the total oxygen uptakes of these two systems are comparable with each other. If the mince had contained any appreciable amount of undamaged tissue the polyphenols in this undamaged portion would not have become oxidized and the total oxygen uptake of the mince would have been lower than that of the infusion, incubated with enzyme. Our mince, therefore, probably approximates to a homogenate, and according to Bennet-Clark (1949) it is a characteristic of plant vacuolate tissue to show negligible total respiration in homogenates.

The total polyphenol content of Ceylon leaf (cf. Lamb, 1936) is of the same order as that of leaf from Assam, so that total uptakes of green leaf minces from the two districts should be comparable. Comparing the published data for oxygen uptakes (Roberts, 1939, Fig. 8, Betjan leaf; and Sreerangachar 1941, Table 5) of Ceylon and Assam leaf, the much lower figures for the Ceylon leaf mince suggest that it contains a much higher proportion of undamaged tissue than minced Assam leaf.

Referring again to the data for Betjan leaf (Roberts, 1939) initial rates of oxygen uptake and carbon dioxide output are 3·7 and 1·2 μl./hr./mg. wet weight. After 1 hr. shaking these rates have fallen to 0·2 μl. oxygen and 0·08 μl. carbon dioxide/hr./mg. wet weight respectively. With leaf disks suspended in water the rate of carbon dioxide output (Deb & Roberts, 1940) is about 0·8 μl. carbon dioxide/hr./mg. wet weight. The leaf samples are reasonably comparable, so that it may be concluded that when polyphenol oxidation is nearly complete the rate of carbon dioxide output has fallen to about 10% of its level in intact tissue. At this stage some polyphenol oxidation is still progressing, so that the data indicate at least 90% inactivation of respiratory activity as a result of the fine mince. The low rate of oxygen uptake in leaf minces, obtained from species low in polyphenols, affords similar evidence, although Sreerangachar (1949) has queried these data and pointed out that the initial rate of oxygen uptake in a mince of *Tropaeolum majus* leaf (Roberts & Sarma, 1940) is of the same order as that recorded by Jensen (1923) for the intact tissue. We consider the initial rate represents a brief phase of irreversible oxidation of carriers and emphasize that after this short initial rapid uptake, lasting not longer than 15 min., the rate of oxygen uptake falls to less than 10% of this level. It is this final low rate which was quoted as evidence for the destruction of respiratory activity by fine mincing.

Sreerangachar (1943, 1949) also suggests that low respiratory rates in leaf minces may be due to inhibition of respiration in intact tissue by polyphenol oxidation products. Our observations (Roberts & Wood, 1951*b*) show, however, that such inhibitions are negligible.

Carbon dioxide production in fine minces

Although it may be considered established that our fine minces contain practically no normally respiring tissue, the possibility remains that carbon dioxide might be formed as a result of oxidation of substances in the leaf by the *o*-quinones produced by enzymic oxidation of the polyphenols.

The fact that there is no inhibition of carbon dioxide output by arsenite, iodoacetate, malonate or 1-naphthol-2-sulphonic acid (cf. Axmacher & Ludwig, 1936), and no increase in carbon dioxide output on addition of cozymase, makes it unlikely

that the carbon dioxide originates from a normal glycolytic breakdown.

In view of the fact that high temperatures of fermentation result in appreciable inactivation of oxidase activity (Roberts, 1941*b*), such high temperature fermentation would be expected to affect the R.Q., as some differential effect on the extent of inactivation of the various enzymes involved would be expected. The absence of such an effect suggests that enzymes other than the oxidase are not involved in carbon dioxide production.

The identity of the R.Q.'s observed for minces and systems composed of enzyme preparation and tea catechins also suggests that the enzymes and coenzymes concerned in glucose breakdown are not involved in the production of carbon dioxide in fermentation, as the acetone-treated tissue, constituting our enzyme preparation, can hardly contain the enzymes and coenzymes required for such glucose oxidation.

Enzymic oxidation of glucose, with *o*-quinones as H-acceptors is therefore probably eliminated, as an explanation of the origin of carbon dioxide in tea fermentation. We have also been unable to find any evidence for direct oxidation of substances such as sugars, amino-acids, etc. when these substances are added to systems producing *o*-quinones. Experiments with infusions of leaf, free or freed from polyphenols, also suggest that, apart from polyphenols of the type found in tea leaf, there are no substances present in such infusions capable of yielding carbon dioxide when oxidized by *o*-quinones.

The evidence available therefore indicates that almost the sole sources of carbon dioxide in fermenting leaf minces are the polyphenols themselves. Roberts & Wood (1950) have established that the polyphenols in tea leaf containing pyrogallol groupings, are oxidized enzymically with production of carbon dioxide. No other source of carbon dioxide in a mince has so far been demonstrated.

SUMMARY

1. The fine minces of tea leaf obtained in this laboratory are almost completely devoid of respiratory activity.

2. Carbon dioxide evolution in such minces is not associated with activity of any of the enzymes concerned with carbohydrate breakdown.

3. Oxidation of substances in the mince other than polyphenols is not detectable, so that carbon dioxide probably originates mainly from polyphenol oxidation.

4. Under factory conditions fermenting leaf in Assam shows little or no respiratory activity.

5. Carbon dioxide production in fermentation in the factory is also largely due to polyphenol oxidation, but there may be other subsidiary sources.

The authors wish to express their thanks to the Chief Scientific Officer, Mr C. J. Harrison, for his continued interest in this work and to the Indian Tea Association for permission to publish these results. Thanks are also due to Dr A. C. Chibnall, F.R.S., for a sample of cozymase.

REFERENCES

Axmacher, F. & Ludwig, H. (1936). *Biochem. Z.* **286**, 1.
Bennet-Clark, T. A. (1949). *Ann. Rev. Biochem.* **18**, 639.
Deb, S. B. & Roberts, E. A. H. (1940). *Biochem. J.* **34**, 1507.
Dixon, M. (1934). *Manometric Methods.* Cambridge University Press.
Jensen, P. (1923). *K. danske vidensk. Selsk. Skr. Biol. Med.* **1**, 34.
Lamb, J. (1936). *Bull. Tea Res. Inst. Ceylon*, **17**, 65.
Nielson, J. P. & Bohart, G. S. (1944). *Industr. Engng Chem.* (Anal. ed.), **16**, 701.
Reichert, R. (1944). *Helv. chim. Acta*, **27**, 961.
Roberts, E. A. H. (1939). *Biochem. J.* **33**, 842.
Roberts, E. A. H. (1941*a*). *Nature, Lond.*, **148**, 285.
Roberts, E. A. H. (1941*b*). *Biochem. J.* **35**, 909.
Roberts, E. A. H. (1941*c*). *Biochem. J.* **35**, 1219.
Roberts, E. A. H. (1942). *Advanc. Enzymol.* **2**, 113.
Roberts, E. A. H. (1949). *Biochem. J.* **45**, 538.
Roberts, E. A. H. & Sarma, S. N. (1938). *Biochem. J.* **32**, 1819.
Roberts, E. A. H. & Sarma, S. N. (1940). *Biochem. J.* **34**, 1517.
Roberts, E. A. H. & Wood, D. J. (1950). *Biochem. J.* **47**, 175.
Roberts, E. A. H. & Wood, D. J. (1951*a*). *Biochem. J.* **49**, 414.
Roberts, E. A. H. & Wood, D. J. (1951*b*). *Biochem. J.* Submitted for publication.
Sreerangachar, H. B. (1941). *Biochem. J.* **35**, 1106.
Sreerangachar, H. B. (1943). *Biochem. J.* **37**, 656.
Sreerangachar, H. B. (1949). *Biochem. J.* **44**, 23.
Wardlaw, C. W. & Leonard, E. R. (1939). *Ann. Bot., Lond.*, **3**, 27.

Studies on Yeast Metabolism

1. FRACTIONATION AND MICRODETERMINATION OF CELL CARBOHYDRATES

By W. E. TREVELYAN and J. S. HARRISON
Research and Development Department, Distillers Co. Ltd., Epsom, Surrey

(*Received* 16 *December* 1950)

Direct measurement of the metabolic changes in the intracellular carbohydrate of yeast has usually been restricted to the determination of total hydrolysable carbohydrate, that is to say, of reducing power after acid hydrolysis of a cell suspension. The work of Pickett & Clifton (1943), or of Fales & Baumberger (1948), exemplifies this technique applied respectively to the aerobic and anaerobic metabolism of glucose by yeast. Clifton (1946) has reviewed studies in some of which indirect methods were used: variation in carbon content, dry weight, and opacity have all been taken as indices of polysaccharide synthesis. Nickerson (1949) has taken the increase of dry weight of yeast during the aerobic metabolism of glucose as measuring, not merely synthesis of cell carbohydrate, but even synthesis of a particular polysaccharide, glycogen. Yeast, however, contains several other carbohydrates (see, for example, Neuberg, 1946): e.g. mannan, trehalose, and a 1:3-glucan insoluble in dilute acid or alkali. Of these trehalose (Pigman & Goepp, 1948), and glucan (Hassid, Joslyn & McCready, 1941) are only hydrolysed by drastic treatment which, when applied to yeast, might cause destruction of less stable sugars, such as the pentose derived from nucleotides and nucleic acids. In consequence, the determination even of total hydrolysable carbohydrate would seem, in principle, unsatisfactory. Schemes for fractionation of some of the constituents of yeast, followed by determination of each as reducing sugar after hydrolysis have been described, the latest being that of Payen (1949). These procedures frequently require large amounts of material and are excessively time-consuming.

By determining carbohydrate colorimetrically with the anthrone reagent (Dreywood, 1946) we have been able to estimate the total carbohydrate of the yeast cell directly, and also to analyse separate fractions without lengthy hydrolysis. Fractionation was achieved by extracting the yeast with trichloroacetic acid, after which the extracted material was treated with alkali, and an alcohol precipitation carried out according to the method used by Good, Kramer & Somogyi (1933) for the determination of glycogen in animal tissue. The residue from these extractions formed a third fraction. Application of paper chromatography enabled this empirical procedure to be related to the known carbohydrate constituents of yeast.

EXPERIMENTAL

Separation of the fractions

Fraction 1. *Trichloroacetic acid extract.* Trichloroacetic acid (TCA) is generally used to extract from yeast, *inter alia*, hexose phosphates (Macfarlane, 1936; Juni, Kamen, Reiner & Spiegelman, 1948). At room temperature and ice-bath temperature 10% (w/v) TCA was found to extract much more carbohydrate from pressed baker's yeast than could be accounted for as phosphoric esters. In fact, when TCA extracts were examined by descending paper chromatography at room temperature, using the method of Partridge (1948), the only sugar detectable by the $AgNO_3$ reagent previously described (Trevelyan, Procter & Harrison, 1950) was trehalose (Fig. 1, *a*). Whatman no. 1 filter paper was used with *s*-collidine (Yorkshire Tar Distillers, purified by

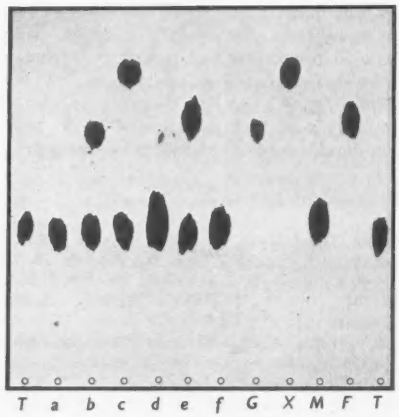


Fig. 1. Filter-paper chromatogram of TCA extract of yeast. *T*, trehalose, $R_F = 0{\cdot}29$; *a*, TCA extract of yeast; *b*, extract + glucose; *c*, extract + xylose; *d*, extract + maltose; *e*, extract + fructose; *f*, extract + trehalose; *G*, glucose, $R_F = 0{\cdot}48$; *X*, xylose, $R_F = 0{\cdot}59$; *M*, maltose, $R_F = 0{\cdot}32$; *F*, fructose, $R_F = 0{\cdot}50$. Sugar spots detected by alkaline silver reagent after developing with collidine.

Br treatment as recommended by Partridge) saturated with water as the solvent. It will be seen from Fig. 1 that TCA does not interfere with the movement of sugars on the chromatogram.

Fraction 2. *Alkali treatment.* Following standard procedure (see Meyer, 1943) for the extraction of glycogen plus mannan from yeast, the TCA-extracted cells were digested with 30% (w/v) aqueous KOH on a water bath at 100° for 15 min., after which 2 vol. ethanol were added and the mixture was refrigerated overnight. The solid (fraction 3) was then centrifuged off, washed with 67% (v/v) ethanol, shaken with water, and again centrifuged. The supernatant,

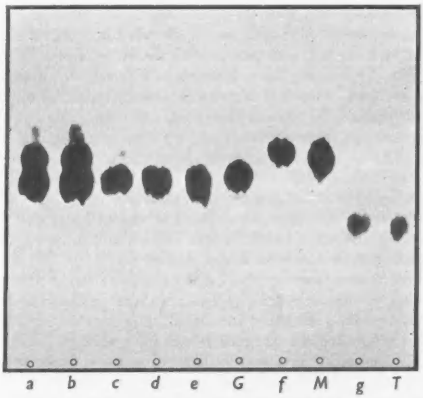


Fig. 2. Filter-paper chromatogram of carbohydrate fractions from yeast: *a*, hydrolysate of mannan + total glycogen, extracted by hot 2N-H_2SO_4 from TCA-extracted yeast; *b*, hydrolysate of mannan + alkali-extractable glycogen; *c*, hydrolysate of glycogen not extracted by alkali, but extracted by acid; *d*, hydrolysate of glucan after removing mannan and total glycogen with acid; *e*, hydrolysate of glucan after first removing alkali-soluble glycogen and mannan, and then acid-soluble glycogen; *G*, glucose, R_F=0·47; *f*, mannan hydrolysate; *M*, mannose, R_F=0·54; *g*, TCA extract; *T*, trehalose, R_F=0·36. Sugar spots detected by alkaline silver reagent after developing with collidine.

containing the water-soluble carbohydrates, was made 2N in H_2SO_4, hydrolysed for 2–3 hr. at 100°, and the residual acid was removed by adding excess $BaCO_3$ and centrifuging. Chromatography of the solution showed the expected glucose and mannose; a faint third spot which was not identified also appeared (Fig. 2, *b*). To separate the mannan from the alkali digest Fehling's solution was added and the mannan-copper complex was centrifuged down, washed with 0·5N-NaOH, dissolved in the minimum amount of HCl, and the mannan reprecipitated with ethanol (Ling, Nanji & Paton, 1925). The hydrolysed material showed a single spot on the chromatogram which was identified as mannose (Fig. 2, *f*). From Fig. 2 it will be seen that no appreciable amount of mannose is present in the yeast after hydrolysis of the separated carbohydrates, except in the mannan fraction.

Fraction 3. *Residue.* The washed residue from the alkali digestion was treated with 60% H_2SO_4 which dissolved the carbohydrate. After standing for some time at room temperature the acid was diluted to about 5N and heated at 100° to effect hydrolysis. Chromatography showed that the hydrolysate contained glucose but no detectable mannose (Fig. 2, *e*). It seemed probable, however, that the glucose originated from a mixture of glycogen and glucan, since heating of the washed residue for 15 min. in the water bath at 100° with 2N-H_2SO_4 extracted a carbohydrate which on further hydrolysis with the same reagent gave glucose only (Fig. 2, *c*). In the light of further evidence presented in the Discussion the carbohydrate must be considered to be glycogen-like, whereas the residue from this treatment required hydrolysis with 60% H_2SO_4 in the cold, and in other ways (see Discussion) showed the properties of glucan.

With modifications on these lines the above methods of fractionation were applied in the scheme of analysis described in the next section. In the subsequent part of this paper the carbohydrate obtained by extracting intact yeast with 10% (w/v) TCA is referred to as 'acid-soluble', that precipitated by Fehling's solution as 'mannan', the residue after treatment with hot 2N-H_2SO_4 as 'glucan', and the carbohydrate which is not accounted for in fractions 2 and 3 as mannan or glucan is called 'glycogen'. This nomenclature agrees with that generally accepted.

Microdetermination of carbohydrate

The analytical procedure of Morris (1948), in which colour is developed by means of the heat produced on mixing anthrone dissolved in 95% H_2SO_4 with the aqueous test solution, proved unsatisfactory. Random fluctuations occurred, the mean of several determinations being reasonably reproducible, but single determinations unreliable. Consistent results were, however, obtained by mixing the reagents in the cold and developing colour by subsequent heating in the water bath. The method differs slightly from that recently described by Seifter, Dayton, Novic & Muntwyler (1950), in that the anthrone is dissolved in acid of minimum strength (i.e. just sufficiently strong to prevent precipitation of anthrone during manipulation) in order to produce as little heat as possible on mixing the reagent with the carbohydrate solution.

Reagent. Conc. H_2SO_4 (500 ml.) was added to 200 ml. of water. Anthrone (0·2 g.) was dissolved in 100 ml. of the diluted acid by thorough shaking, any undissolved particles being removed from the surface with a pipette. The reagent should be prepared fresh daily.

Procedure. Anthrone reagent (5 ml. portions) was pipetted into 1 in. diameter Pyrex tubes, which were then cooled in ice water and 1 ml. of solution, containing 10–100 μg. of glucose or equivalent carbohydrate, was layered on top of the acid. When all tubes had been prepared they were removed from the bath and the contents mixed by rapid swirling: the temperature of the mixture rises slightly above room temperature. After covering with close-fitting bakelite bottle-tops the tubes were heated for exactly 10 min. in a water bath, which must be boiling vigorously since the time and intensity of heating are important (Seifter *et al.* 1950). Bakelite lids were chosen because, owing to their physical properties, interference from condensation water was avoided. The tubes were then immediately plunged into running tap water and cooled for at least 2 min. It was found convenient to use a wire basket for holding the tubes.

The green colour was read on the Unicam D.G. spectrophotometer at 620 mμ. against a H_2SO_4 blank (2 vol. H_2SO_4 : 1 vol. water) using ⅝ in. diameter tubes.

Standards. Glucose standard curves obtained by plotting colour measurement against amount of glucose, were satisfactorily linear between 25 and 100 μg., intermediate readings departing from those calculated from a 100 μg. standard by no more than ±1 μg. A standard containing 100 μg. of glucose, together with a distilled water blank, was included in each batch of tubes. The standard was prepared daily from a 0·1% (w/v) solution of glucose in saturated benzoic acid. Mannose gives only 55% of the colour produced by an equal amount of glucose, and hence, if a measure of the amount of mannan, as such, is required it is preferable to include a mannose standard in the appropriate batch. For biochemical studies we find it convenient to express all constituents in terms of glucose. Reproducibility is good, the mean difference between parallel determinations on any of the fractions from a given yeast suspension being about 1%.

The scope of the anthrone reaction, and its connexion with carbohydrate structure, has been dealt with by Sattler & Zerban (1948). We find that pentoses, free or combined as in ribonucleic acid or adenosinetriphosphate, give a colour after about 3 min. heating, which fades rapidly after this time (cf. Seifter *et al.* 1950). However, some residual absorption at 620 mμ. is always found. Seifter and co-workers have dealt with interference from certain non-carbohydrate compounds; one other important interfering substance likely to be present in fermentation studies is hydrazoic acid. In $2·5 \times 10^{-3}$ M azide the colour given by 100 μg. of glucose is only one-third of normal, an effect which is not surprising in view of the wide range of organic compounds attacked by HN_3 in the presence of mineral acid (Schmidt reaction: see Wolff, 1946). The HN_3 was removed by 5 min. aeration of samples which had been acidified with H_2SO_4. In general, we have found that oxidizing compounds have a profound effect on the anthrone reaction, whereas reducing substances such as SO_2 or $SnCl_2$ appear to have little effect. Some evidence has been obtained suggesting that the enol form of anthrone is the effective reagent.

Carbohydrates of yeast: application of fractionation procedure

The procedure described was worked out for the analysis of a 0·5% suspension of yeast in water or buffer, as used by us in manometric experiments, but it can easily be adapted to suit other requirements. Throughout this work analyses are based on the original weight of yeast used in the fermentation experiments (see below), because the conditions were such that, although there was no significant increase in cell numbers, changes occurred in the dry weight of the yeast due to synthesis or utilization of intracellular carbohydrate. Thus 0·5% suspension refers in all cases to a concentration equivalent to 0·5 g. of pressed yeast (24–26% dry matter at the commencement of the test) suspended in a total volume of 100 ml. When centrifuging small quantities (about 10 mg.) of yeast the cells sediment much better from potassium phosphate or phthalate buffer (0·067 M, pH 4·5) than from water. The baker's yeast we have studied contains regularly about 80 μg. of total carbohydrate per mg. as glucose (i.e. 1 mg. of yeast gives the same colour in the anthrone reaction as 80 μg. of glucose).

Total carbohydrate. The yeast suspension was diluted to 1 mg./ml.: if the analysis was not to be performed at once, the dilution was made with H_2SO_4 to a final concentration of 0·1–0·5 N to prevent metabolic change. Analysis was carried out as described under Procedure above. When material was limited, instead of adding 1 ml. of suspension directly, a smaller volume, say 0·2 ml., was added to the anthrone reagent, followed by 0·8 ml. of water. On mixing the anthrone reagent and yeast suspensions a slight turbidity was noticeable, but this disappeared on heating.

Extracellular sugar. Samples (*a*) 2 ml. and (*b*) 4 ml. of the 0·5% yeast suspension were centrifuged in 15 ml. round-bottomed tubes and 1 ml. samples of the supernatant were used undiluted for analysis as described under Procedure.

Acid-soluble sugar. The cell residues from tubes (*a*) and (*b*) were washed with 2 ml. of phosphate buffer, the washings being discarded. The cells were suspended in 1 ml. of 10% TCA and shaken continuously at room temperature for 30 min. Prolonging the extraction to 3 hr. did not increase the amount of carbohydrate extracted. Then 1·5 ml. of water was added to each tube and, after centrifuging, the supernatants from tubes (*a*) and (*b*) were combined by decanting into a single stoppered 10 ml. measuring cylinder. The cells were washed with 2 ml. of water because residual TCA would produce chloroform in the subsequent treatment with alkali. Washings were added to the cylinder and the contents diluted to 10 ml. Samples of this solution were used for analysis as described under Procedure.

Low concentrations of TCA do not interfere in the anthrone reaction, but 10% TCA induces precipitation of anthrone when the tubes are cooled, though colour development is unaffected. If much larger quantities of yeast are used, the extraction step should be repeated to ensure complete removal of the acid-soluble material.

Mannan. To tube (*a*) 0·5 ml. of 30% KOH was added, the tube was covered loosely with a glass stopper, and heated for 30 min. on the water bath at 100°. After cooling to about 40°, 0·5 ml. of Fehling's solution was added, and on shaking the mannan-copper complex flocculated. This was allowed to stand for some time, and was then centrifuged, washed with 1 ml. of 0·5 N-NaOH, using a glass rod to break up the precipitate, and recentrifuged. After draining off the liquid 1 drop of 6 N-HCl was added to the precipitate, followed immediately by 1·4 ml. of water. Copper was dissolved and the precipitate became colourless; 1·4 ml. of ethanol was then added. After mixing and standing overnight in the refrigerator the solid was centrifuged and washed with 2 ml. 50% (v/v) ethanol. Washings were discarded and the tube drained. The remaining drop of ethanol was removed by standing the tube in a water bath at 100° for 1 min. and aspirating air through the tube. A reagent (5 ml.) consisting of a suspension of about 0·1% $BaSO_4$ in 0·04 N-H_2SO_4 was added and the tube was stoppered and vigorously shaken. On centrifuging the $BaSO_4$ assisted complete sedimentation of the insoluble material. The supernatant contained the mannan and was analysed without further treatment as described under Procedure.

It should be noted that the cell debris still contains glycogen (see above), and therefore, if larger quantities of yeast are used for analysis, it is preferable to separate the 30% KOH extract in tube (*a*) from the residue before the copper-precipitation step. However, quantitative studies show that this portion of the carbohydrate remains insoluble throughout the procedure.

Glycogen plus mannan. The contents of tube (*b*) were heated with KOH as described for the estimation of mannan,

and 1·4 ml. of ethanol were added to the hot mixture. Shaking caused precipitation of the polysaccharides, and the tube was then immersed in the water bath at 100° until the ethanol began to boil. The tube was placed in the refrigerator overnight, after which it was centrifuged, the supernatant was discarded, and the solid washed with dilute ethanol as in the mannan determination. 2 N-H_2SO_4 (2 ml.) was added and the tube was heated on the water bath at 100° for 15–30 min. After cooling, 3 ml. of the H_2SO_4:$BaSO_4$ reagent were added. The supernatant after centrifuging was poured into a 10 ml. cylinder, the residue was washed by resuspension in water and centrifuging, and the combined supernatants were made to 10 ml. This solution contained mannan, together with glycogen extractable by alkali, and that part of the glycogen not extracted by alkali but dissolved from the cell residue by acid. Total glycogen was measured by difference between this and the mannan figure.

If the H_2SO_4 extraction step is omitted, the alkali-extractable glycogen may be similarly determined after treating the ethanol-precipitated polysaccharides directly with the H_2SO_4 reagent as in the mannan determination or, more easily, by measuring the carbohydrate in the alkali digest directly by the anthrone reaction after diluting with water and centrifuging.

Glucan. The mixture of $BaSO_4$ and cell residue remaining was well drained, and 4 ml. of anthrone reagent were added, agitating well with a glass rod which was splayed out at the end. After centrifuging, the supernatant containing the glucan was poured off into a dry tube and 1 ml. was mixed with 4 ml. of anthrone reagent, cooled, and colour developed after mixing with 1 ml. of water.

Application to fermentation studies

The procedure outlined above works well when the yeast suspension is not actively fermenting. If it is desired to analyse samples which have just been withdrawn from active fermentation, the danger of change in composition during the repeated washing necessary to remove external fermentable sugar is always present. It has been found possible, when glucose is being used as substrate, to stop the fermentation by adding TCA, and thus to obtain an acid-soluble extract containing trehalose, any sugar phosphates present, and residual glucose. The phosphoric esters are generally present in quite minute amounts (Macfarlane, 1936) and may be disregarded in the present scheme of analysis. The glucose may then be removed by hypobromite oxidation, when the trehalose remaining is directly determined. (For consideration of such oxidations see Pigman & Goepp, 1948.)

Removal of glucose. To about 5 ml. of TCA extract, 5 N-NaOH was added until neutral to phenolphthalein (external indicator: phenolphthalein produces a strong orange colour with H_2SO_4 in the anthrone reaction), then 0·5 ml. of saturated bromine water, followed by 1 ml. of 0·5 N-NaOH. After 15 min. incubation at 30°, 0·2 ml. of bromine water was added and incubation was continued for a further 15 min. The mixture was acidified with 0·5 ml. of 4 N-H_2SO_4, made to 10 ml. and excess bromine was removed by aeration. This treatment completely removed at least 4 mg. of glucose. Trehalose was determined on the solution.

RESULTS

An idea of reproducibility of the analyses obtained in actual fractionations can be obtained from Table 1 where two pairs of results are given. In Table 1, column *A*, results are given for a fractionation after incubation of 10 mg. of yeast for 2 hr. at 30° in 0·067 M-KH_2PO_4: column *B* gives results obtained in a separate experiment with 10 mg. of the same yeast. Columns *C* and *D* give the results of two experiments after similar incubation of 10 mg. of yeast in the presence of 2 mg. of glucose. The values obtained in the fractionation of successive yeast samples agree well, the mean difference being 10 μg./10 mg. yeast. The maximum difference of 40 μg. occurs in the total carbohydrate figures, in these examples, and represents a percentage variation from the mean of about $\pm 3\%$. The sum of the separate fractions is in all cases rather less than the total determined directly.

Table 1. *Carbohydrate analyses of fractions obtained from yeast*

(Columns *A* and *B* are fractionations from two experiments in each of which 10 mg. of a sample of pressed baker's yeast (25% dry matter) was incubated for 2 hr. at 30° in 0·067 M-KH_2PO_4. Columns *C* and *D* are from similar fractionations where 2 mg. of glucose was added to the medium.)

	Carbohydrate (expressed as μg. of glucose)			
Fraction	*A*	*B*	*C*	*D*
Total carbohydrate	830	790	1198	1160
Extracellular	5	11	51	49
Acid-soluble	233	226	246	246
Mannan	190	190	229	224
Glycogen	175	174	381	387
Glucan	158	159	176	171
Sum of fractions	761	760	1083	1077

Analyses of the alkali-soluble portion (fraction 2), of mannan, and of glycogen (after dilute acid treatment) showed that only 70% of the glycogen was extracted by alkali. In 10 mg. of unfermented yeast the 190 μg. glucose equivalent of mannan represents 346 μg. of mannose, giving a total carbohydrate value of some 96 μg. of hexose per mg., or about 38% of the total dry matter. The actual weight of di- and poly-saccharide would, of course, be somewhat less.

DISCUSSION

The methods described for the fractionation of yeast carbohydrates are essentially empirical, although based on the accepted techniques for separating these compounds; but they give satisfactorily reproducible results, and have been applied successfully to a study of yeast fermentation presented in a further communication (Trevelyan, Gammon, Wiggins & Harrison, 1952). One striking observation in this work was that during fermentation of glucose the greatest variations occurred in fraction 3 (residue), suggesting that part of this fraction had biochemically the properties of glycogen (for reviews of the definition and chemistry of glycogen see Meyer, 1943; Bell, 1948). Furthermore, the amount of glucose-yielding material extracted by alkali varied with the proportion of yeast to alkali, although reproducible quantitative results were

obtained by a given procedure. It would therefore appear that the glycogen may not have been completely extracted by the alkali treatment, particularly in view of the fact that various workers have shown that yeast glycogen is not homogeneous. Ling *et al.* (1925) refer to two forms of glycogen, one 'a constituent of the plasma' and readily extractable with water from dried yeast, and the other 'more intimately connected with the cell wall'. McAnally & Smedley-Maclean (1937) showed that the residue after extraction with 60% potassium hydroxide contained magnesium and phosphate, and digestion of the residue with cold N-hydrochloric acid rendered water-soluble a polysaccharide resembling glycogen. Jeanloz (1944) purified yeast glycogen which was separated by electrodialysis into two fractions, one soluble, the remainder (73%) insoluble in water: some indication that yeast glycogen was partly phosphorylated was obtained. Alkali digestion was not used for preparation 'car il n'est pas possible d'effectuer le broyage total de toutes les cellules'.

In order to decide whether in our method the extraction of glycogen by alkali was complete a method for distinguishing between glycogen and glucan was necessary. Chromatography of hydrolysates does not, of course, differentiate. The periodate staining method of Hotchkiss (1948) was, therefore, used to follow the effect of alkali treatment, since he reported that yeast glucan, lacking an α-glycol grouping except at the ends of chains, would probably give little colour in the staining reaction (cf. Barry & Dillon, 1943). It was found that the yeast cell stained generally with the reagent, although a darker reticulated structure appeared in the cell interior. Extraction with trichloroacetic acid did not alter this appearance. Heating from 15 min. to 3 hr. at 100° with 30% (w/v) potassium hydroxide gave a picture of a surrounding unstained cell wall, with some stained material remaining in the interior of the cell, but separated from the wall. This was presumably glycogen, since potassium hydroxide had been shown to extract the mannan readily. Heating of the cell residue for 15 min. at 100° with 2N-sulphuric acid, however, left a residue which did not stain. The acid extract contained a carbohydrate which, on further hydrolysis, gave glucose only (Fig. 2, *c*). The residue gave the anthrone reaction and hydrolysed to glucose, and hence was presumably glucan (Fig. 2, *d*). While this paper was in course of preparation, a publication by Bell & Northcote (1950) appeared which provided confirmation of some of these findings. Whether the non-extractability of part of the glycogen by strong alkali denotes any sharp chemical or physiological difference, or whether alkali treatment, by depositing insoluble inorganic products inside the cell wall, mechanically prevents further diffusion of part of the glycogen, cannot be decided on the evidence so far obtained.

SUMMARY

1. The anthrone reaction has been adapted to give reproducible quantitative results, and has been applied to the determination of the total carbohydrate of baker's yeast.

2. An empirical procedure for fractionating the cell carbohydrates is described which may be applied to as little as 10 mg. of yeast (2·5 mg. dry matter).

3. Paper chromatography and the periodate staining method of Hotchkiss have been used to relate the fractions to the known constituents of yeast, namely. trehalose, glycogen, mannan and glucan.

4. Digestion with alkali (30% potassium hydroxide) does not bring all the glycogen into solution, and a further treatment with acid is necessary.

5. A modification of the procedure is described which enables yeast cell carbohydrates to be determined in actively fermenting suspensions.

The authors thank the Directors of the Distillers Co. Ltd. for permission to publish this paper.

REFERENCES

Barry, V. C. & Dillon, T. (1943). *Proc. R. Irish Acad.* **49** B, 177.
Bell, D. J. (1948). *Biol. Rev.* **23**, 256.
Bell, D. J. & Northcote, D. H. (1950). *J. chem. Soc.* p. 1944.
Clifton, C. E. (1946). *Advanc. Enzymol.* **6**, 269.
Dreywood, R. (1946). *Industr. Engng Chem.* (Anal. ed.), **18**, 499.
Fales, F. W. & Baumberger, J. P. (1948). *J. biol. Chem.* **173**, 1.
Good, C. A., Kramer, H. & Somogyi, M. (1933). *J. biol. Chem.* **100**, 485.
Hassid, W. Z., Joslyn, M. A. & McCready, R. M. (1941). *J. Amer. chem. Soc.* **63**, 295.
Hotchkiss, R. D. (1948). *Arch. Biochem.* **16**, 131.
Jeanloz, R. (1944). *Helv. Chim. Acta*, **27**, 1501.
Juni, E., Kamen, M. D., Reiner, J. M. & Spiegelman, S. (1948). *Arch. Biochem.* **18**, 387.
Ling, A. R., Nanji, D. R. & Paton, F. J. (1925). *J. Inst. Brew.* **31**, 316.
McAnally, R. A. & Smedley-Maclean, I. (1937). *Biochem. J.* **31**, 72.
Macfarlane, M. G. (1936). *Biochem. J.* **30**, 1369.
Meyer, K. H. (1943). *Advanc. Enzymol.* **3**, 109.
Morris, D. L. (1948). *Science*, **107**, 254.
Neuberg, C. (1946). *Ann. Rev. Biochem.* **15**, 435.
Nickerson, W. J. (1949). *Experientia*, **5**, 202.
Partridge, S. M. (1948). *Biochem. J.* **42**, 238.

Payen, R. (1949). *Canad. J. Res.* **27**, 749.
Pickett, M. J. & Clifton, C. E. (1943). *J. cell. comp. Physiol.* **21**, 77.
Pigman, W. W. & Goepp, R. M. (1948). *Chemistry of the Carbohydrates.* New York: Academic Press.
Sattler, L. & Zerban, F. W. (1948). *Science*, **108**, 207.
Seifter, S., Dayton, S., Novic, B. & Muntwyler, E. (1950). *Arch. Biochem.* **25**, 191.
Trevelyan, W. E., Gammon, J. N., Wiggins, E. H. & Harrison, J. S. (1952). *Biochem. J.* **50**, 303.
Trevelyan, W. E., Proctor, D. P. & Harrison, J. S. (1950). *Nature, Lond.*, **166**, 444.
Wolff, H. (1946). *Organic Reactions*, **3**, 307.

Studies on Yeast Metabolism

2. SYNTHESIS OF CELL CARBOHYDRATES DURING GLUCOSE FERMENTATION, AND ITS INHIBITION BY AZIDE

By W. E. TREVELYAN, J. N. GAMMON, E. H. WIGGINS and J. S. HARRISON
Research and Development Department, Distillers Co. Ltd., Epsom, Surrey

(*Received* 16 *December* 1950)

The major products of the fermentation of glucose by yeast are ethanol and carbon dioxide, but under certain conditions an appreciable amount of the glucose metabolized is accounted for by synthesis of cellular carbohydrate (see review by Clifton, 1946). The nature, extent and status of carbohydrate assimilation appear, however, to be still undecided. This is partly due to the use of indirect methods of judging the extent of carbohydrate synthesis using, for example, cell carbon content, dry matter, opacity or, in many cases, relying simply on manometric data. We have therefore examined the applicability of the method of analysis of yeast carbohydrates previously described (Trevelyan & Harrison, 1952) to this problem, and further extended the method to an examination of the action of azide on fermentation, as this inhibitor has played an important part in assimilation studies.

METHODS

Overall carbohydrate balance after fermentation

Fermentations were carried out in an atmosphere of N_2 at 30° in the Warburg manometric apparatus. Carbohydrates were determined (Trevelyan & Harrison, 1952) on the yeast suspension prior to fermentation, and on the suspensions remaining in the flasks after exhaustion of fermentable sugar.

Pressed baker's yeast (Distillers Co. Ltd.) was suspended in 0·067 M-KH_2PO_4 at pH 4·5. The substrate, in most cases glucose, was added from the side arm of the Warburg flask after temperature equilibration, and NaN_3, when used, was dissolved in the substrate solution. The solubility of CO_2 in the medium was taken to be the same as in water, although according to graphical data presented by Van Slyke, Sendroy, Hastings & Neill (1928) the solubility in 0·067 M-KH_2PO_4 at 38° is about 3% less than in water. This variation hardly affects our figures.

In anaerobic yeast fermentation CO_2 is formed by the decarboxylation of a C_3 compound, pyruvic acid, which is split to give CO_2 and a C_2 moiety. The nature or fate of the latter does not affect the applicability of the Gay-Lussac equation, according to which 1 mg. of hexose is equivalent to almost exactly 250 μl. of CO_2 at N.T.P. This value is used to calculate the 'hexose equivalent' of the CO_2 measured manometrically. Carbohydrate analyses are expressed 'as glucose', i.e. in terms of the colour produced in the anthrone reaction by a standard glucose solution.

Active fermentation studies

In studies of the changes occurring during fermentation, samples were withdrawn at different stages of active fermentation of glucose by yeast, and the progressive variations with time of some constituents of the yeast suspension were determined. As explained in a previous paper (Trevelyan & Harrison, 1952) all analyses are referred to the original weight of pressed yeast (24–26% dry matter).

Procedure A. Yeast suspension (yeast suspension is defined in Part 1) and glucose solution were prepared separately and chilled in ice. When cold, equal volumes were mixed and 2 ml. of the mixture were dispensed into each of six 15 ml. centrifuge tubes. Air was rapidly displaced from the tubes by N_2, and they were stoppered and shaken in a water bath at 30°. (For details of amounts of yeast and glucose, see under separate experiments.)

Procedure B. This procedure was used to study fermentation in the presence of NaN_3. Portions (1 ml.) of a yeast suspension were measured into centrifuge tubes, which were equilibrated at 30° while nitrogen was passed through the tubes. Glucose-azide solution, previously brought to 30°, was added and the tubes were stoppered and shaken. At various times after the commencement of fermentation tubes were removed, immediately centrifuged, and residual glucose determined by the anthrone method (Trevelyan & Harrison, 1952). In some experiments the ethanol content of the centrifugate was determined, using the microdiffusion method of Macleod (1949). Glycerol was estimated, after removing residual glucose by precipitation with $CuSO_4$ and $Ca(OH)_2$ according to Van Slyke (1917), by periodate oxidation and colorimetric determination of the resultant formaldehyde by the method described by Corcoran & Page (1947) for the estimation of mannitol in physiological fluids.

In repeat experiments, after withdrawing the tubes from the water bath, fermentation was arrested by the addition of trichloroacetic acid (TCA) to a final concentration of approx. 10% (w/v), and estimations of (*a*) TCA-extractable carbohydrate after bromine oxidation, and (*b*) total polysaccharide after KOH digestion of the extracted cells, were carried out as previously described (Trevelyan & Harrison, 1952). Production of CO_2 was followed on yeast-glucose preparations of identical concentration in a separate manometric experiment. In all cases the fermentation mixture was 0·067M with respect to KH_2PO_4. The results, then, include analytical determinations carried out on samples from the same block of yeast (stored in the refrigerator) and used for centrifuge-tube fermentations performed over several days, and also manometric data for the same yeast. These results, although obtained under slightly varying conditions, may, because of their reproducibility and congruity, be taken as applying to a single fermentation without great error.

RESULTS

Autofermentability of yeast carbohydrate

In agreement with the results of Spiegelman & Nozawa (1945), it was found that yeast, in the absence of external substrate, metabolized its carbohydrate reserves very slowly. Carbohydrate analysis suggested that a slight fall in glycogen content, of the same order as the CO_2 output, occurred after 2 hr. (Table 1, Exp. 1). In a parallel experiment, in which $1·25 \times 10^{-3}$M-NaN_3 was added, the analysis at 2 hr. was the same as in its absence. Azide, therefore, did not promote endogenous fermentation (cf. Reiner & Spiegelman, 1947).

finding has already been discussed (Trevelyan & Harrison, 1952). Application of the complete analytical fractionation (Table 1, Exp. 2) showed that in these experiments it is justifiable to treat the change in yeast carbohydrate occurring during glucose fermentation as mainly due to synthesis of glycogen. However, it cannot be assumed without further study that the same happens, for example, during the fermentation of higher concentrations of sugar: indeed, the mere fact of the occurrence in yeast of other forms of carbohydrate shows that their synthesis must take place. The sum of the separate carbohydrate fractions was less than the directly measured total carbohydrate, and the discrepancy increased after fermentation. The significance of this is not yet clear.

The values obtained for mannan by direct colorimetric comparison with glucose standards have to be multiplied by 1·82 to correct for the lower chromogenic value of mannose. However, because of the relatively small change in this fraction under our experimental conditions, changes in the total carbohydrate figure may be taken as a measure of variation of the hexose of the yeast.

By-products of glucose fermentation

In overall balance experiments, the discrepancy between the recorded CO_2 output and that calculated from the glucose fermented, which we shall term the glucose defect, was due to side reactions of which carbohydrate synthesis was only one. No more than 40% of the glucose defect was accounted for as synthesized cell carbohydrate (Table 1, items 11 and 12). The remainder was only ascribable in part to glycerol formation (Fig. 1). In one experiment 2 mg. of glucose were completely fermented by 10 mg. of yeast, and lactic acid was

Table 1. *Yeast carbohydrate* (*a*) *before addition of substrate, and* (*b*) *after complete fermentation of* 10 *mg. of pressed yeast* (*about* 2·5 *mg. dry weight*) *in* 2·00 *ml. of* 0·067M-KH_2PO_4 *at* 30°

(All analytical values expressed as μg. glucose.)

Exp. no.	1		2		3		4		5		6	
Substrate (glucose) (μg.)	0		2000		2000		2000		2000		2000	
NaN_3	0		0		0		0		$5·0 \times 10^{-5}$M		$2·5 \times 10^{-3}$M	
	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)
(1) Total carbohydrate	849	830	899	1198	871	1053	758	1037	758	801	797	457
(2) Extracellular	13	5	5	51	8	20	7	15	7	18	15	7
(3) TCA extract (trehalose)	243	233	238	246	226	255	164	199	164	126	168	44
(4) Total polysaccharide	577	523	604	786	585	679	518	665	518	510	509	415
(5) KOH extract	—	—	—	—	223	231	—	—	—	—	219	188
(6) Glycogen	217	175	211	381	—	—	—	—	—	—	—	—
(7) Mannan	195	190	217	229	167	170	183	202	183	183	188	154
(8) Glucan	165	158	176	176	—	—	—	—	—	—	—	—
(9) Sum of fractions (2)–(4). Fermentation time (min.)	833	761	847	1083	819	954	689	879	689	654	692	466
	—	120	—	95	—	100	—	100	—	100	—	160
(10) CO_2 (as hexose)	—	16	—	1427	—	1423	—	1495	—	1792	—	1968
(11) Substrate not converted to CO_2 (glucose defect)	—	—	—	573	—	577	—	505	—	208	—	32
(12) Increase in total carbohydrate	—	−19	—	+299	—	+182	—	+279	—	+43	—	−340

Glycogen synthesis during glucose fermentation

When 2 mg. of glucose were completely fermented by 10 mg. of yeast the total carbohydrate increased, due largely to an increase in the polysaccharide fractions (Table 1, Exps. 2–4). Only part of this material was extracted from the yeast cell by KOH treatment: the significance of this

determined in the medium by the method of Barker & Summerson (1941): only 37 μg. of lactic acid were produced. It is probable that several other compounds are formed, each in relatively small amount, as was found for oxidative metabolism of yeast by Pickett & Clifton (1943). The contribution of carbohydrate synthesis to the glucose defect may vary from yeast to yeast: but it is clearly not justifiable

to interpret manometric findings in terms of carbohydrate synthesis alone unless actual analysis is first used to establish the properties of the particular organism studied.

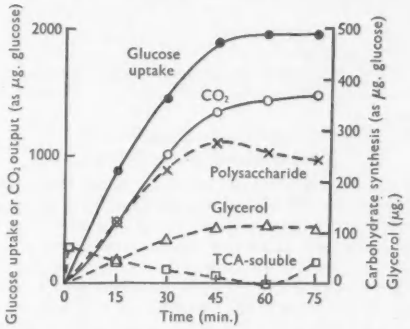


Fig. 1. Fermentation of 2 mg. of glucose by 10 mg. of yeast in 1 ml. of 0·067 M-KH_2PO_4 at 30° (Procedure *A*, Exp. 7).

Fermentability of glycogen in presence of substrate

In Exp. 7 polysaccharide (glycogen) was formed up to 45 min. in amount proportional to the CO_2 output (Fig. 1). After 45 min. the fermentation rate fell away owing to exhaustion of substrate. Glycogen actually decomposed, but the rate of destruction decreased rapidly as the external glucose level fell. This suggested that yeast can ferment its endogenous carbohydrate as long as external substrate is present. This interpretation is supported by a consideration of the results of Reiner, Gest & Kamen (1949), who showed that external glucose accelerated the turnover of cell products which had been synthesized by yeast from radioactive glucose. A kinetic interpretation of glycogen formation during fermentation is clearly indicated, i.e. the reaction

glycogen + inorganic phosphate $\rightleftharpoons$ glucose-1-phosphate

is fully reversible as long as external substrate is present, the direction of the overall reaction depending upon the concentration of the various components.

Formation of intermediates during fermentation

The curves for CO_2, polysaccharide and glycerol formation (Exp. 7, Fig. 1) are of similar form, and the amount of each of these products at any stage of the fermentation is roughly proportional. If other by-products, as yet unidentified, behave similarly, the implication is that the CO_2 output at any stage of the fermentation should be a constant fraction of the glucose uptake. In the case studied, this fraction increased from 0·54 after 20 min. to 0·75 at 75 min. This was probably due to the formation within the yeast cell of intermediates of the Meyerhof cycle in concentrations which were, after a rapid initial formation, in equilibrium with the external glucose concentration and consequently decreased to zero as the substrate was removed by the fermentation (cf. Fales & Baumberger, 1948). A similar explanation may be adduced for the peculiar variation in carbohydrate extracted by TCA (Fig. 1). Even at ice-bath temperature carbohydrate resistant to hypobromite oxidation was formed; this material could scarcely have been trehalose, and presumably represented such intermediates as fructose phosphates and dihydroxyacetone phosphate. The terminal increase in TCA-extractable carbohydrate, however, was probably due to genuine trehalose formation.

Percentage conversion of glucose to CO_2

In agreement with Fales & Baumberger (1948) we have found that CO_2 production does not entirely cease when external glucose has been exhausted. The percentage conversion of glucose to CO_2, calculated from manometric data, thus depends somewhat upon the time after disappearance of external substrate at which the experiment is stopped. If

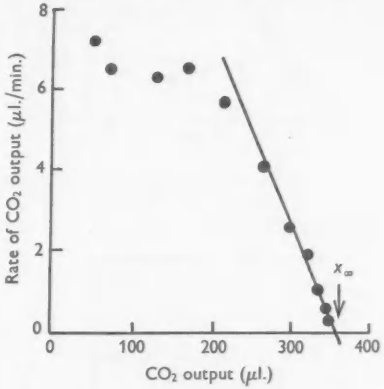


Fig. 2. Rate of fermentation of 2 mg. of glucose by 10 mg. of yeast in 2 ml. of 0·067 M-potassium hydrogen phthalate, pH 4·5 at 30° (Exp. 8).

the velocity of gas production is plotted against the total CO_2 evolved, as in Fig. 2, it is found that, as the external glucose concentration falls, the curve approximates to a straight line. We define the 'CO_2 output' (x_∞) as the intercept on the 'CO_2 volume' axis obtained by extrapolating the straight line portion of this curve.

Action of azide on cell carbohydrate

When fermentation of glucose was carried out in the presence of $2·5 \times 10^{-3}$ M-NaN_3, the CO_2 evolved was equivalent to the complete conversion of added glucose to CO_2 and ethanol. Carbohydrate analysis (Table 1, Exp. 6), however, showed that part of the yield was due to the metabolism of cell carbohydrate, trehalose in particular, though polysaccharide (probably glycogen) also decreased. Fig. 3 shows that the equivalence of glucose uptake, CO_2 output, and ethanol production applied approximately throughout the fermentation. Trehalose and polysaccharide of the yeast decreased (Fig. 3), and the rate of disappearance of these compounds fell as the external substrate was removed, in accordance with the finding that azide, in the absence of substrate, did not promote endogenous metabolism. Glycerol formation, though decreased as compared with the control, was still appreciable. The decomposition of trehalose in a similar fermentation was confirmed by chromatographic methods previously described (Trevelyan & Harrison, 1952). Yeast was incubated for 90 min. at 30°: (*a*) anaerobically

without substrate, (*b*) aerobically with glucose, (*c*) anaerobically with glucose, and (*d*) anaerobically with glucose and $2{\cdot}5 \times 10^{-3}$M-NaN_3, after which the cells were washed with water and extracted with 10% (w/v) trichloroacetic acid.

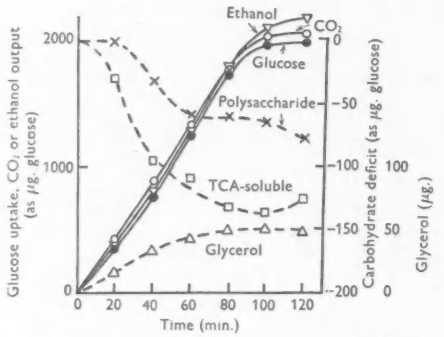


Fig. 3. Fermentation of 2 mg. of glucose by 10 mg. of yeast in 1 ml. of 0·067 M-KH_2PO_4 and $2{\cdot}5 \times 10^{-3}$M-NaN_3 at 30° (Procedure *B*, Exp. 9).

Fig. 4. Filter-paper chromatogram of sugars extracted from yeast after incubation at 30° for 90 min. *G*, glucose, $R_F = 0{\cdot}48$; 1, after anaerobic metabolism of glucose in the presence of $2{\cdot}5 \times 10^{-3}$M-NaN_3, showing some unfermented glucose; 2, after anaerobic metabolism of glucose; 3, after aerobic metabolism of glucose; 4, after anerobic incubation with water; *T*, trehalose, $R_F = 0{\cdot}28$. Sugar spots detected by alkaline silver reagent after developing with collidine.

The extracts were examined chromatographically with the results shown in Fig. 4, where glucose and trehalose were used as markers. The amount of trehalose was of the same order in tests (*a*), (*b*) and (*c*), but in the presence of azide (*d*) the amount was much smaller, indicating breakdown of trehalose during fermentation of the glucose.

Fig. 3 suggests that in the last 20 min. of the fermentation a conversion of polysaccharide to trehalose occurred. In Exp. 9 (Fig. 3), throughout the fermentation the extracellular carbohydrate was almost entirely glucose, for after hypobromite oxidation the following values for non-glucose carbohydrate were obtained in successive 20 min. periods: 23, 5, 2, 5, 9, 23 μg. (as glucose)/ml. The initial glucose concentration was 2000 μg./ml. Thus the disappearance of trehalose promoted by $2{\cdot}5 \times 10^{-3}$M-NaN_3 was not due to a permeability change allowing the diffusion of trehalose into the medium, and its subsequent hydrolysis and re-absorption.

Effect of different concentrations of azide on carbohydrate synthesis

With a concentration of NaN_3 of 5×10^{-5}M, the trehalose and polysaccharide content of yeast was almost unchanged by the complete fermentation of 2 mg. of glucose (Table 1, Exp. 5). Table 2 shows that lower concentrations of azide allowed synthesis of cell carbohydrate, whereas higher concentrations brought about a net decomposition. Over the range $0\text{–}15 \times 10^{-5}$M-$NaN_3$, increase in CO_2 output was largely due to the effect on carbohydrate synthesis; the plot of glucose defect against increase in cell carbohydrate was approximately linear, with a slope of 1·1. Stated in more conventional terms, this means that in higher concentrations of azide part of the CO_2 originated from the fermentation of preformed cell carbohydrate.

Table 2. *Effect of* NaN_3 *on the fermentation of 2 mg. of glucose by 10 mg. of yeast*

NaN_3 concentration (M $\times 10^{-5}$)	Glucose defect, i.e. glucose not converted to CO_2 (μg.)	Increase in total yeast carbohydrate expressed as glucose (μg.)	Initial rate of CO_2 production (μl./min.)
Exp. 10. Medium 0·067 M-KH_2PO_4 (pH 4·5)			
0	528	223	8·00
1·0	456	189	8·33
2·5	296	38	9·16
5·0	232	−2	9·01
7·5	212	−49	8·58
15·0	164	−72	8·33
Exp. 11. Medium 0·067 M-potassium hydrogen phthalate (pH 4·5)			
0	600	259	6·83
1·0	356	23	8·83
2·5	188	−78	7·92

Omission of phosphate from the medium greatly increased the effect of a given concentration of azide (Table 2, Exp. 11). Such an effect would seem to be contrary to the known effect of azide in preventing phosphate uptake during fermentation (Spiegelman, Kamen & Sussman, 1948): however, the addition of azide together with substrate, as used in our experiment, may explain this, as we have observed that azide added to a yeast suspension before glucose had a more pronounced inhibitory effect.

Effect of azide on fermentation rate

Azide concentrations below $2{\cdot}5 \times 10^{-5}$M. As the conversion of glucose to CO_2 is raised from 73% in the absence of azide to 86% in the presence of $2{\cdot}5 \times 10^{-5}$M-NaN_3, the rate of CO_2 production is increased in exact proportion to the increase of CO_2 output. Because of a decrease in carbohydrate synthesis the rate of glucose uptake is not significantly affected.

Azide concentrations between $2{\cdot}5 \times 10^{-5}$ M *and* $2{\cdot}5 \times 10^{-3}$ M. The rate of glucose consumption is decreased, the inhibitory action of azide on this rate being greater at the beginning of the fermentation than near the end. This has the effect of decreasing the slope of the lower part of the curve obtained by plotting CO_2 production against time, with the result that the curve becomes nearly linear over a greater part of the fermentation period at higher azide concentrations.

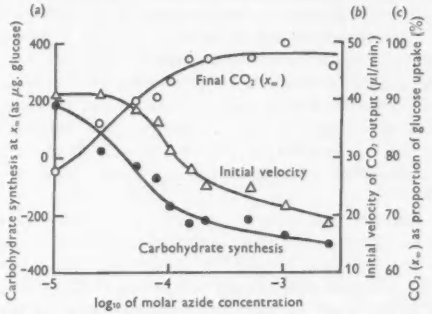


Fig. 5. Effect of azide on (*a*) carbohydrate synthesis, (*b*) initial rate of CO_2 output, and (*c*) final volume of CO_2 (x_∞), during the fermentation of 2 mg. of glucose by 10 mg. of yeast in 1 ml. of 0·067 M-KH_2PO_4 at 30° (Exp. 12). ●—●, Carbohydrate synthesis when the CO_2 evolved $= x_\infty$; △—△, initial velocity of CO_2 output calculated between 5 and 30 min.; ○—○, final CO_2 evolved, x_∞, expressed as a percentage of glucose used.

When (*a*) the amount of carbohydrate synthesized, (*b*) the initial rate of fermentation, as CO_2 output per min., and (*c*) the final CO_2 volume (x_∞), as a percentage of the glucose used, are plotted against the logarithm of the azide concentration, sigmoid curves are obtained (Fig. 5). The curves for fermentation in phosphate-free medium (not shown) are similar, but displaced towards the region of low azide concentration; the slope of the linear part of the curves is approximately the same as when phosphate is present.

Effect of azide on phosphate metabolism

Spiegelman *et al.* (1948) have stated that in their experiments the inorganic phosphate of yeast decreased progressively as fermentation proceeded, and that this effect was almost entirely repressed by $2{\cdot}5 \times 10^{-3}$ M-NaN_3. They interpreted this as showing that 'azide can effectively prevent the flow of phosphate from the inorganic to the organic fraction which accompanies the fermentation of glucose'. However, Mirski & Wertheimer (1939) showed that in glucose fermentation the internal inorganic phosphate dropped rapidly on addition of glucose, the lowest value being recorded after 1–2 min., and that as fermentation progressed the inorganic phosphate recovered almost to the initial level. We have suggested above, that the intermediates of the Meyerhof cycle are extremely labile and rapidly reach equilibrium with the external sugar concentration. Thus, if the latter is decreased by centrifugation of the yeast followed by washing with water, the internal inorganic phosphate of the yeast cell will increase (cf. Dische, 1940). As it is not entirely clear how the measurements of Spiegelman *et al.* were arrived at, or whether precautions were taken to prevent decomposition of labile phosphate during analysis, we have studied the effect of $2{\cdot}5 \times 10^{-3}$ M-NaN_3 on the inorganic phosphate of yeast during fermentation of glucose (2 mg./ml.) by yeast (10 mg./ml.) suspended in 0·017 M-potassium succinate buffer (pH 4·5). Fermentation was stopped by adding TCA to a final concentration of about 5% (w/v), and after extraction at ice-bath temperature the inorganic phosphate, in TCA solution, was determined by the method of Berenblum & Chain (1938). The fermentation was carried out at 30° in centrifuge tubes using procedure *B*. Results are shown in Fig. 6. The variations in inorganic phosphate level during azide-free fermentation followed the course described by Mirski & Wertheimer (1939). With $2{\cdot}5 \times 10^{-3}$ M-NaN_3, however, the inorganic phosphate of the suspension actually increased. Now, although the fermentation rate in the presence of azide was much less than that of the control (initial rate 34% of the control), some formation of phosphorylated intermediates must have occurred for fermentation to proceed at all. Hence the rise in inorganic phosphate must have been due to the preponderance of decomposition of a pre-existing phosphorus compound of the yeast other than orthophosphate, over a simultaneous formation of labile phosphorylated intermediates. Hence, the interpretation of the effect of azide on the inorganic phosphate of yeast, as due to an interference with fermentative phosphorylation, is not justified.

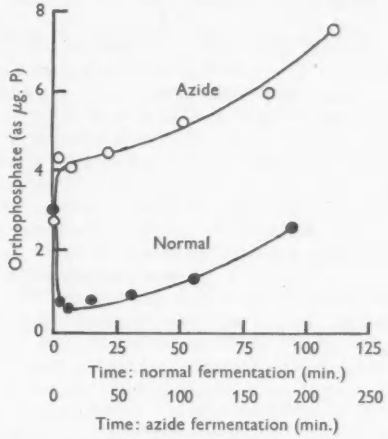


Fig. 6. Orthophosphate content of fermentation mixtures after fermenting 2 mg. of glucose with 10 mg. of yeast in 1 ml. of potassium succinate buffer (pH 4·5) at 30° with and without the addition of NaN_3 (Procedure *B*, Exp. 13). Different time scales used for normal and azide experiments because of different fermentation rates. ●—●, Normal; ○—○, with $2{\cdot}5 \times 10^{-3}$ M-NaN_3.

DISCUSSION

Synthesis of cell carbohydrate in yeast fermentation

Under our experimental conditions, the synthesis of cell carbohydrate is largely accounted for as glycogen formation. There is no reason to suppose that the

synthesis of glycogen in yeast requires any other mechanism than that shown to operate in the separate isolated enzyme systems (see, for example, Colowick & Sutherland, 1942). Kiessling (1939), using yeast enzyme, showed that equilibrium was reached in the reaction

$$\text{glycogen} + \text{orthophosphate} \rightleftharpoons \text{glucose-1-phosphate}$$

at a definite value of the ratio [glucose-1-phosphate]/[orthophosphate]. Later workers, using muscle and plant phosphorylases (cf. Cori, Cori & Green, 1943), established the dependence of this ratio on pH: in particular, the ratio is about 0·2 at pH 6·4, which may be taken as approximately the internal pH of the yeast cell (Conway & O'Malley, 1946). Thus a consideration of *in vitro* findings leads to the conclusion that glycogen synthesis in yeast would occur when the ratio [glucose-1-phosphate]/[orthophosphate] inside the cell is greater than 0·2.

It is interesting to note that Sussman, Spiegelman & Reiner (1947) have reported manometric experiments which they interpret as showing that carbohydrate synthesis is repressed when the pH of the medium is increased from 4·5 to 8·5, and suggest a direct effect upon the internal phosphorylase equilibrium as being responsible. As it seems unlikely that the overall internal pH of the cell would be much affected by the external pH (cf. the treatment of phosphate uptake by Hevesy, 1947), such explanations should be accepted with caution.

Nickerson (1949) has suggested that in living yeast metaphosphate is formed concurrently with glycogen, and that the insolubility of the former drives the phosphorylase reaction to the side of glycogen synthesis. Table 2 (Exp. 13) shows that in the absence of external phosphate 259 μg. of cellular carbohydrate were formed after fermentation of 2 mg. of glucose. Formation of an equivalent amount of metaphosphate would bind about 60 μg. of phosphorus, i.e. twenty times the orthophosphate phosphorus present in the yeast before fermentation. In the absence of further evidence, it would seem that Nickerson's mechanism is implausible. Similarly, quantitative considerations rule out the treatment of glycogen synthesis as an important dephosphorylating mechanism (Brockmann & Stier, 1947), for the small amount of glycogen formed would remove only about 6 % of the adenosinetriphosphate bonds generated by fermentation.

We consider, therefore, that glycogen synthesis during fermentation is due to (*a*) the production of ester phosphate, and (*b*) the decrease of intracellular orthophosphate to a low level. As glucose becomes exhausted, the orthophosphate rises: thus eventually a point should be reached when the conditions favour the breakdown of glycogen. To some extent this has been shown to occur towards the end of fermentation, but the rate of breakdown rapidly falls to a negligible value when substrate has disappeared from the medium. This point is clearly connected with the virtual non-fermentability of cell carbohydrate in the absence of external substrate. It is likely that a kinetic explanation is required, connected with the findings of Dische (1940) that the 'labile pyrophosphate' (presumably adenosinetriphosphate), formed rapidly on the addition of glucose to yeast suspensions, was capable of spontaneous destruction by enzymes at speeds approaching that of fermentation, with the result that, as the concentration of glucose decreased during fermentation, the amount of the phosphorylated fraction fell away. The enzyme concerned in the breakdown of 'labile pyrophosphate' was presumably adenosinetriphosphatase (Meyerhof, 1945).

Indirect evidence of the action of adenosinetriphosphatase during fermentation is provided by the heat produced during fermentation (Winzler & Baumberger, 1938) which may be due to the wastage of high-energy phosphate bonds rather than their use in synthesis. If the breakdown of polysaccharide into hexosemonophosphate is slow relative to the action of adenosinetriphosphatase, the resulting level of adenosinetriphosphate in the cell may become so low as to limit the overall speed of the endogenous fermentation. Alternatively, the level of other important phosphorylated compounds may fall, such as that of the coenzyme of phosphoglucomutase (i.e. glucose-1:6-diphosphate).

Action of azide on glycogen synthesis

It seems certain that a given level of azide will have widely different inhibitory powers on fermentation by different yeasts, and this should be borne in mind when comparing the results reported here with those of other investigators. Azide interferes with several aspects of the anaerobic metabolism of yeast; e.g. adaptive enzyme formation (Spiegelman, 1947), carbohydrate assimilation (Reiner & Spiegelman, 1947), and exchange of internal and external phosphate (Spiegelman *et al.* 1948). These last authors consider that the fundamental action of azide is in reducing the supply of energy-rich phosphate normally generated during fermentation. An analogy with the action of arsenate on the triose phosphate dehydrogenase system (Warburg & Christian, 1939) has been assumed, although *in vitro* experiments designed to show such an effect were negative. Reiner (1948), however, suggested that the action of azide differed from that of arsenate, and was unlikely to be due simply to the suppression of high-energy phosphate generation.

We have suggested that synthesis of glycogen by living yeast will occur when the ratio [glucose-1-phosphate]/[orthophosphate] inside the cell exceeds a certain critical value, and that fermentation of

intracellular carbohydrate takes place (as long as external substrate is present) when the ratio falls below the equilibrium ratio. In normal fermentation the critical ratio appears to be attained at a very low level of external glucose concentration, and consequently in the complete fermentation of milligram amounts of glucose under the conditions of our experiments the overall change in the level of internal cell carbohydrate is not greatly affected by the usage of the polysaccharides of the cell. It is probable that the effect of azide on carbohydrate synthesis may be explained on the same basis, i.e. as primarily due to (*a*) a fall in glucose-1-phosphate concentration, or (*b*) a rise in orthophosphate concentration.

(*a*) *Diminution in glucose-1-phosphate.* Since low concentrations of azide reduce glycogen formation without alteration in the rate of glucose uptake, a general lowering of high-energy phosphate cannot initially occur, for the rate of the hexokinase reaction with a given amount of enzyme depends on the concentration of glucose at the enzyme surface, which must have been unaltered, and the concentration of adenosinetriphosphate, which therefore also was presumably unaffected. However, a specific lowering of the concentration of glucose-1-phosphate may occur, analogous to the action of arsenate on the phosphorylase of *Pseudomonas saccharophila* (Doudoroff, Barker & Hassid, 1947) and of potato (Katz, Hassid & Doudoroff, 1948). Here arsenate competes with phosphate and brings about the breakdown of sucrose and starch respectively to free glucose via an unstable glucose-1-arsenate.

(*b*) *Rise in orthophosphate.* We have already considered reasons why the rise in orthophosphate induced by $2{\cdot}5 \times 10^{-3}$M-sodium azide during fermentation in phosphate-free medium suggests the breakdown of a phosphate compound other than the intermediates of the Meyerhof cycle. This may be metaphosphate, since Wiame (1949) states that the synthesis of metaphosphate, which requires phosphate, potassium ions, and substrate, is suppressed by concentrations of azide which do not interfere with fermentation.

Either mechanism would explain the competition between azide and (internal) inorganic phosphate which is suggested by the influence of addition of extracellular phosphate in our experiments. Such a competition probably accounts for the finding that the inhibitory action of azide on fermentation rate is greater in the initial stages of fermentation, since the orthophosphate content of the cell rises as fermentation proceeds. The effect of higher concentrations of azide on rate of fermentation would be explainable as due to a drain of high-energy phosphate which could clearly occur by either of mechanisms (*a*) or (*b*). Mechanism (*b*) would also explain the potentiation by azide of the inhibitory action of fluoride on fermentation (Spiegelman *et al.* 1948), since the inhibition of enolase by fluoride depends on the concentration of orthophosphate (Warburg & Christian, 1942). As both mechanisms are outside the main reaction chain of the fermentation cycle, the failure of Spiegelman *et al.* (1948) to demonstrate azide inhibition in cell-free extracts or *in vitro* enzyme systems is explicable.

The breakdown of trehalose induced by higher concentrations of azide suggests that trehalose, like glycogen, participates inside the yeast cell in an equilibrium with some intermediate of the Meyerhof cycle, possibly glucose-1-phosphate.

SUMMARY

1. The changes in total carbohydrate, trehalose, glycogen, mannan and glucan of baker's yeast occurring during glucose fermentation have been determined.

2. Under the experimental conditions employed cellular carbohydrate increased during fermentation; this was mainly due to synthesis of glycogen.

3. About 70% of the glucose metabolized was converted to carbon dioxide and ethanol. Less than half of the remainder was accounted for as synthesized cell carbohydrate.

4. Sodium azide inhibited carbohydrate synthesis. The magnitude of the effect depended upon the concentration of azide: synthesis occurred below $2{\cdot}5 \times 10^{-5}$M, whereas greater concentrations promoted breakdown of glycogen and trehalose. With $2{\cdot}5 \times 10^{-3}$M-sodium azide, carbon dioxide and ethanol formed were equivalent to 100% conversion of fermented glucose, but partly originated by fermentation of preformed cell carbohydrate.

5. The mechanism of glycogen synthesis has been discussed in terms of the known properties of the enzymic reactions of fermentation.

6. The action of azide upon glycogen synthesis during fermentation has been discussed and possible mechanisms of action considered.

The authors thank the Directors of the Distillers Co. Ltd. for permission to publish this paper.

REFERENCES

Barker, S. B. & Summerson, W. H. (1941). *J. biol. Chem.* **138**, 535.
Berenblum, I. & Chain, E. (1938). *Biochem. J.* **32**, 215.
Brockmann, M. C. & Stier, T. J. (1947). *J. cell. comp. Physiol.* **29**, 159.
Clifton, C. E. (1946). *Advanc. Enzymol.* **6**, 269.
Colowick, S. P. & Sutherland, E. W. (1942). *J. biol. Chem.* **144**, 423.
Conway, E. J. & O'Malley, E. (1946). *Biochem. J.* **40**, 59.
Corcoran, A. C. & Page, I. H. (1947). *J. biol. Chem.* **170**, 165.
Cori, C. F., Cori, G. T. & Green, A. A. (1943). *J. biol. Chem.* **151**, 39.
Dische, Z. (1940). *C.R. Soc. Biol., Paris*, **133**, 380.
Doudoroff, M., Barker, H. A. & Hassid, W. Z. (1947). *J. biol. Chem.* **170**, 147.
Fales, F. W. & Baumberger, J. P. (1948). *J. biol. Chem.* **173**, 1.
Hevesy, G. (1947). *Advanc. Enzymol.* **7**, 111.
Katz, J., Hassid, W. Z. & Doudoroff, M. (1948). *Nature, Lond.*, **161**, 96.
Kiessling, W. (1939). *Biochem. Z.* **302**, 50.
Macleod, L. D. (1949). *J. biol. Chem.* **181**, 323.
Meyerhof, O. (1945). *J. biol. Chem.* **157**, 105.
Mirski, A. & Wertheimer, E. (1939). *Enzymologia*, **7**, 58.
Nickerson, W. J. (1949). *Experientia*, **5**, 202.
Pickett, M. J. & Clifton, C. E. (1943). *J. cell. comp. Physiol.* **21**, 77.
Reiner, J. M. (1948). *Arch. Biochem.* **19**, 218.
Reiner, J. M., Gest, H. & Kamen, M. D. (1949). *Arch. Biochem.* **20**, 175.
Reiner, J. M. & Spiegelman, S. (1947). *J. cell. comp. Physiol.* **30**, 347.
Spiegelman, S. (1947). *J. cell. comp. Physiol.* **30**, 315.
Spiegelman, S., Kamen, M. D. & Sussman, M. (1948). *Arch. Biochem.* **18**, 409.
Spiegelman, S. & Nozawa, M. (1945). *Arch. Biochem.* **6**, 303.
Sussman, M., Spiegelman, S. & Reiner, J. M. (1947). *J. cell. comp. Physiol.* **29**, 149.
Trevelyan, W. E. & Harrison, J. S. (1952). *Biochem. J.* **50**, 298.
Van Slyke, D. D. (1917). *J. biol. Chem.* **32**, 455.
Van Slyke, D. D., Sendroy, J., Hastings, B. & Neill, J. M. (1928). *J. biol. Chem.* **78**, 765.
Warburg, O. & Christian, W. (1939). *Biochem. Z.* **303**, 40.
Warburg, O. & Christian, W. (1942). *Biochem. Z.* **310**, 384.
Wiame, J. M. (1949). *J. biol. Chem.* **178**, 919.
Winzler, R. J. & Baumberger, J. P. (1938). *J. cell. comp. Physiol.* **12**, 183.

The Preparation and Properties of Aucubin, Asperuloside and Some Related Glycosides

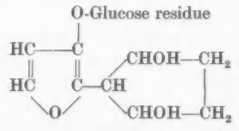
By A. R. TRIM and R. HILL
Biochemical Laboratory, University of Cambridge

(*Received* 14 *February* 1951)

Some of the many types of glycosides which have been described have been thought to be specific products of narrow taxonomic groups of plants. However, more and more of them are proving to occur widely in the plant kingdom. The work described in this paper shows that aucubin, asperuloside and related glycosides, which are probably derived from substituted furan aglycones, constitute one such widely occurring type.

Aucubin was first isolated by Bourquelot & Hérissey (1905) from the leaves of *Aucuba japonica* (family Cornaceae). It is a monohydrate of a monoglucoside of an unstable aglucone, which these workers were unable to isolate or identify. Asperuloside was first isolated by Hérissey (1925*a*, *b*, 1926*a*, *b*, 1927, 1933) from several members of the family Rubiaceae. He showed that it, too, is a monoglucoside of an unstable aglucone, which, like aucubin, loses 1 mol. of water when heated to 105–110°.

Karrer & Schmid (1946) suggested for aucubin the substituted furan structure (I) which explains the properties described by Bourquelot & Hérissey (1905) and Bergmann & Michalis (1927). This



(I)

glucoside has now been isolated from plants belonging to the following families: Cornaceae (Bourquelot & Hérissey, 1905), Garryaceae (Hérissey & Lebas, 1910), Plantaginaceae (Bourdier, 1907), Orobanchaceae (Bridel, 1929), Globulariaceae (Zellner, 1934), Scrophulariaceae (Braecke, 1923*a*), and Eucommiaceae (Plouvier, 1944).

In the present work an improved method for the isolation of glycosides (Hill & Van Heyningen, 1951) has been applied to the preparation of aucubin and asperuloside, and both glycosides can now be prepared in quantity and in a pure form. Aucubin has been obtained from the leaves of *Aucuba japonica* and *Melampyrum arvense* and, for the first time, from *Buddleia globosa* (family Loganiaceae). Asperuloside has been isolated from *Galium aparine*, varieties of *Rubia tinctorum* and, for the first time, from *Daphniphyllum macropodum* (family Euphorbiaceae). New chemical properties of the two glucosides are described, some of which support the suggestion of Hérissey (1925*a*) that aucubin and asperuloside have a similar structure, while others sharply distinguish the two glycosides. There seems to be little evidence that these two glycosides are derived from coumarin, as stated by Armstrong & Armstrong (1931). It has also been shown that other, less well known, glycosides could be conveniently placed in a group with aucubin and asperuloside.

EXPERIMENTAL

All melting points quoted from the authors' observations are corrected.

The preparation of the glycosides and their derivatives

Glycosides and other substances are adsorbed from aqueous solutions by charcoal. Sugars, salts and some other substances are removed from the adsorbed mixture by washing with water. The glycosides remain on the charcoal and are eluted with aqueous ethanol, from which some of them may be easily obtained crystalline.

The preparation of aucubin from Aucuba japonica, Melampyrum arvense *and* Buddleia globosa. Fresh leaves of *A. japonica* (1200 g.) were chopped into small pieces and added to 2·5 l. of boiling water containing 3 g. $CaCO_3$. Boiling was continued for 3 hr. The cooled extract was poured off, the leaves squeezed in a coarse cloth to remove as much fluid as possible and the combined extract filtered through kieselguhr. Animal charcoal (20 g.), previously boiled for about 10 min. with distilled water to remove air, was stirred with the filtrate for 15 min. and then filtered off with kieselguhr. Charcoal (500 g.), boiled as before, was stirred with the filtrate for 15 min. adsorbing most of the aucubin. The charcoal was then filtered off with a large quantity of kieselguhr and the pad washed with 700 ml. distilled water. The adsorbed glucoside was eluted by washing with 7 l. 50% (v/v) ethanol, the eluate reduced to 200 ml. *in vacuo* in the presence of a little $CaCO_3$, filtered and then reduced *in vacuo* to 100 ml. The aucubin crystallized on standing at room temperature: it was filtered off, redissolved in the minimum volume of hot 90% (v/v) ethanol, boiled for a few minutes with animal charcoal, filtered hot and recrystallized twice from 90% ethanol giving 20 g. aucubin, m.p. 176–177°. This material (14 g.) recrystallized three times from 90% ethanol yielded 11·6 g. of aucubin, m.p. 180–181°, $[\alpha]_D^{18°}$ – 164·7° (*l*, 2; *c*, 1·5 in water).

The same method was used to prepare aucubin from *Melampyrum arvense* and *Buddleia globosa*. Yields and constants are recorded in Table 1.

Samples of aucubin hexa-acetate and the two monobromo-hexa-acetyl aucubins *A* and *B* were prepared, the former by the method of Karrer & Schmid (1946) and the two latter by the method of Bergmann & Michalis (1927).

The preparation of asperuloside from Rubia tinctorum *and* Daphniphyllum macropodum. The fresh tips (1730 g.) of the young shoots of *Rubia tinctorum* were extracted with 1400 ml. of 0·33 N-HCl in four equal batches. The shoots were gently pounded in a pestle and mortar until reduced to a pulp, stood at room temperature for 30 min. and then squeezed through coarse calico. The extract was filtered with kieselguhr.

The filtrate was stirred for 10 min. with 30 g. of previously boiled charcoal. The charcoal was removed by filtration with kieselguhr and the filtrate stirred with a further 100 g. charcoal for 10 min. A large bulk of kieselguhr was added and the suspension filtered on a Büchner funnel, washed with 300 ml. distilled water and the glycoside eluted with 3 l. 50% ethanol. The eluate was reduced to 150 ml. *in vacuo* in the presence of 2 g. $CaCO_3$. Ethanol (150 ml. of 95%) was added and the light gelatinous precipitate removed by filtration through kieselguhr. The filtrate was reduced to a syrup by distillation *in vacuo* and dried in a desiccator over $CaCl_2$. The glucoside was extracted from the residue with hot ethanol, filtered and reduced to a small volume *in vacuo*. The fine white crystals of asperuloside obtained on standing at 0° were filtered off, washed with a little ice-cold ethanol, recrystallized twice from 95% ethanol and dried in air; 10 g. of asperuloside with the following constants were obtained; m.p. 125–127°, $[\alpha]_D^{18°}$ – 204° (*l*, 2; *c*, 1·7 in water) for the anhydrous glucoside, solubility in water 4% at 18°, 0·2% at 0°; C, 50·6; H, 5·5%; N, S and halogens were absent. Calc. for $C_{17}H_{24}O_{11}$: C, 50·6, H, 5·7% and a molecular weight of 404, Hérissey (1925*a*) found a molecular weight of 409 by the cryoscopic method.

Table 1. *Yields and constants of aucubin*

Source	Method of preparation	Amount of material (g. fresh wt.)	Amount of purified aucubin (g.)	M.p.	$[\alpha]_D$
Aucuba japonica seeds	Bourquelot & Hérissey (1905)	500	15	181°	– 164·9° at 18°
Aucuba japonica leaves	Charcoal adsorption	1200	16·5	181°	– 164·5° at 18°
Melampyrum arvense foliage	Charcoal adsorption	330	2·9	181°	– 165° at 16°
Buddleia globosa leaves	Charcoal adsorption	200	0·7	182°	– 166° at 17°
Plantago lanceolata unripe fruit	Karrer & Schmid (1946)	2000	20	182·3°	– 162° at 15°

Table 2. *Yields of asperuloside*

Source	Method of preparation	Amount of material: Fresh wt. (g.)	Amount of material: Dry wt. (g.)	Amount of asperuloside (g.)	M.p.
Galium aparine seedlings	Hérissey (1926*c*)	—	8980	4·5	126–127°
Galium aparine young shoot tips	Charcoal adsorption	600	Approx. 90	6·5	125–127°
Rubia tinctorum young shoot tips	Charcoal adsorption	1730	Approx. 260	10·0	125–127°
Daphniphyllum macropodum mature leaves	Charcoal adsorption	315	—	2·0	126–129°

This method was used to prepare asperuloside from *Galium aparine* and *Daphniphyllum macropodum*, for leaves of which we are indebted to Mr J. Gilmour, Director of the Royal Horticultural Society's Garden. The purified product from *Daphniphyllum* had the following constants: m.p. 126–129°, mixed m.p. with the sample from *Rubia tinctorum* 126–129°; $[\alpha]_D^{18°}$ – 196 (*l*, 2; *c*, 1·64 in water), for anhydrous material – 200°, C, 50·7; H, 5·6%.

The samples for elementary analysis were recrystallized from wet ethyl acetate, dried in the air for 2 days and then to constant weight over $CaCl_2$ in an atmospheric desiccator. A sample from *Daphniphyllum* lost the equivalent of 0·5 mol. of water of crystallization, one from *Rubia tinctorum* lost the equivalent of 1 mol. These materials were used for analysis. However, if dried further by heating at 110° to constant weight both samples appear to lose a further 0·5 mol. of water. Yields are recorded in Table 2.

Acetyl asperuloside. A crystalline acetyl derivative of asperuloside was obtained by the procedure described by Karrer & Schmid (1946) for the preparation of aucubin hexa-acetate. Crystalline asperuloside (2·1 g.) was heated to 37° for 24 hr. with 12·8 ml. anhydrous pyridine and 10·7 ml. acetic anhydride. Then, following the original procedure in detail, 2·7 g. of the acetyl derivative, m.p. 153°, were obtained. The melting point could not be raised by further recrystallizations. The compound is soluble in ethanol, methanol, methanol-water mixtures and ethyl acetate and is readily crystallized from these solvents. (Found: C, 53·7; H, 5·4 mol.wt. by the camphor method 757; $C_{33}H_{40}O_{19}$ requires C, 53·5; H, 5·4%; and mol.wt. 740.) Assuming that the molecular weight of anhydrous asperuloside is 404, from the empirical formula, these constants correspond to an octa-acetate, calculated mol.wt. 740. This could not be checked by the determination of acetyl groups by the standard methods which employ acid hydrolysis. It was also impossible to resort to saponification because the glucoside itself reacts with dilute alkali to produce an indeterminate amount of acid.

Some properties and reactions of aucubin and asperuloside

Ultraviolet absorption spectra. The ultraviolet absorption spectrum of asperuloside was determined in both neutral and alkaline solutions. The curves are shown in Fig. 1, together with that for aucubin.

General reactions. Neither aucubin nor asperuloside give colour reactions with $FeCl_3$ and they do not reduce Fehling's solution. Both substances reduce Tollens's $AgNO_3$ reagent slowly in the cold and in a few seconds on heating to 100°. Asperuloside reacts more readily than aucubin. The rate of reaction at 100° is comparable with that of the phenolic glucoside salicin. The chromogenic structures of both substances are stable to alkali and their characteristic colour reactions still occur after boiling with N-NaOH. Karrer & Schmid (1946) showed that aucubin could be recovered after boiling with saturated $Ba(OH)_2$ for 1 hr. Although strong alkali does not affect the capacity of asperuloside to give its colour reactions with acids, dilute alkali causes a rapid, irreversible change in its structure and acid is produced. The

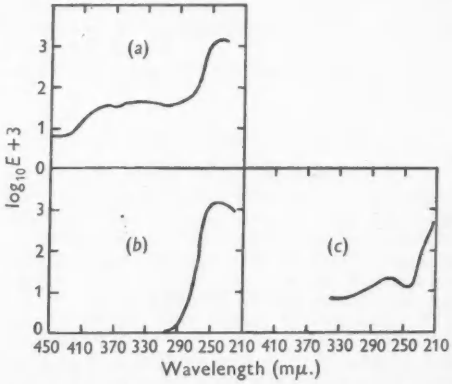


Fig. 1. Absorption spectra of (*a*) solution of 10·95 mg. asperuloside in 100 ml. 0·1 N-NaOH, allowed to stand at room temperature for 2 hr. before measurement. (*b*) Solution of 12·85 mg. asperuloside, recrystallized from water, in 100 ml. water. (*c*) Solution of 27·1 mg. aucubin in 100 ml. water.

accompanying change in the ultraviolet absorption spectrum is shown in Fig. 1. 6·05 and 7·55 mg. of asperuloside were allowed to stand for 5 min. with 5 ml. N/70-NaOH. The remaining NaOH was titrated with N/70-HCl, 3·45 and 3·25 ml. being required respectively, using phenolphthalein as an indicator. Solutions of the pure glucoside are neutral, so that this corresponds to the release of 1·3–1·4 equiv. of acid per g.mol. of glucoside. More acid is produced on standing. When alkali is first added the solution becomes yellow but later this colour fades. A crystalline product could not be obtained from asperuloside after treatment with NaOH.

The action of bromine. Aqueous and alcoholic solutions of aucubin and asperuloside rapidly decolorize Br_2 solutions.

Bergmann & Michalis (1927) have shown that three bromine substitution products of aucubin hexa-acetate are obtained by the action of Br_2 in the cold. Asperuloside acetate does not take up Br_2 in the cold and does so only very slowly on heating.

The action of acids. Aucubin and asperuloside are acid labile and produce coloured acid-degradation products. If the reaction takes place in glacial acetic acid containing a small amount of Cu^{++}, clear blue solutions are obtained. A convenient reagent (reagent *A*) may be prepared by mixing 10 vol. of glacial acetic acid, 1 vol. of 0·2% solution of $CuSO_4.5H_2O$ and 0·5 vol. of conc. HCl.

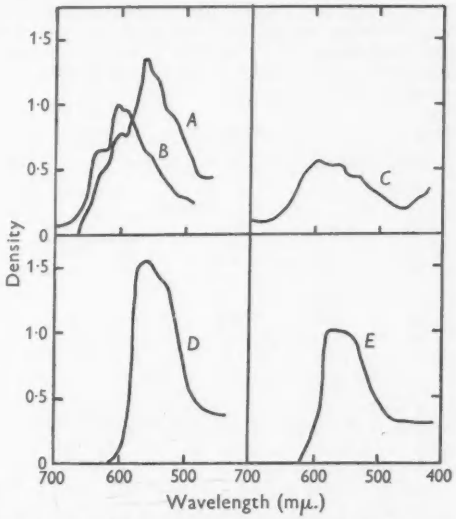


Fig. 2. Absorption spectra of pigments obtained by heating the following mixtures of substances, contained in 1 ml. of water, with 11 ml. of reagent *A* for 5 min. on a boiling-water bath: *A*, 0·488 mg. asperuloside and 4·94 mg. fructose: *B*, 0·488 mg. asperuloside; *C*, 0·432 mg. aucubin; *D*, 0·55 mg. asperuloside brominated in ethanol and 4·94 mg. fructose; *E*, 0·848 mg. bromo-hexa-acetyl aucubin and 4·94 mg. fructose.

If asperuloside (0·5 mg.) is heated to boiling with 2–3 ml. of reagent *A* for 0·5–1 min. a stable, bright-blue colour develops. The same colour develops when the glucoside is heated with the reagent in a boiling-water bath for 3–5 min. with occasional shaking. The cooled solution has two well defined absorption bands, a narrow band I with a centre and maximum at 645 mμ. and a broader band II with a centre and maximum at 600 mμ. This spectrum, measured in a Hilger-Nutting Visual Spectrophotometer, is shown in Fig. 2.

Bands I and II of the blue derivative obtained from asperuloside appear to be characteristic of two forms of the glucoside. If the glucoside is obtained by recrystallization from 90% ethanol, band I is weak or absent and band II is proportionately stronger. If the glucoside is crystallized from water, band I is strong and band II is proportionately weaker. In the latter case the relative strengths of the two bands may be reversed by the addition of a little ethanol to the glucoside before heating with reagent *A*. After the action of alkali on asperuloside, band I is strong and band II is very weak.

Aucubin boiled for 0·5–1 min. with reagent *A* gives a blue of a different tint. There is a single spectral absorption band at 590–600 mμ. (see Fig. 2). Aqueous solutions of aucubin heated with reagent *A* in a boiling-water bath frequently give a green colour, but solid aucubin always gives the blue colour just described.

The effect of other substances on the development of colour from asperuloside, aucubin and their derivatives by the action of reagent A. The colour produced by the action of reagent *A* upon the two glucosides and their derivatives is affected by other substances, particularly by ketohexoses, pyrroles, indoles and phenols. In the examples discussed below colour tests were made by heating both the added substance alone, and the added substance plus glucose, with reagent *A*. In some cases colour changes were observed, but these were relatively weak and distinctly different from the colours obtained with the two glucosides and their derivatives.

The effect of ketoses. The presence of large quantities of aldoses has no effect on the colour developed by heating aucubin, asperuloside and their derivatives with reagent *A*. Ketohexoses cause a shift of absorption towards the blue and the solution assumes a red colour proportionate to the amount of sugar added. With asperuloside and its derivatives this effect is marked. With aucubin it is not so strong. The effect with hexa-acetyl aucubin is exceptional in that the colour becomes olive-green, but there is a marked reddening with the two monobromo-acetyl aucubins. The absorption spectra of some of the pigments produced by fructose are shown in Fig. 2. In each case the molecular concentration of glucoside was approximately the same. The following ketohexoses were tested with asperuloside, brominated asperuloside and monobromo-hexa-acetyl aucubin A and gave a well defined red reaction: fructose, L-sorbose, D- and L-psicose, as the diacetone derivatives, which are hydrolysed to the free sugar during the reaction, and D-tagatose. Sucrose, raffinose, stachyose and irisin, which contain combined fructose, also gave the reaction.

The ketopentoses D- and L-adonose (erythro-2-ketopentose), regenerated from the *o*-nitrophenylhydrazones, did not produce red colours. The ketotetrose, L-erythrulose, regenerated from the *o*-nitrophenylhydrazone, suppressed the development of colour from asperuloside. It gave a brick-red colour with asperuloside, previously treated with with Br_2 in ethanolic solution, and a bright-red colour with bromo-hexa-acetyl aucubin A. Aldoses, dihydroxyacetone, glyceraldehyde and pyruvate had no effect.

This reaction shows the similarity between aucubin and asperuloside. It provides a useful specific test for ketohexoses in the presence of aldoses and other substances and offers the basis of a method for their estimation. Under the above conditions, about 20 mol. of ketose per mol. of glucoside must be added to convert the whole of it to the red pigment. With a fixed amount of asperuloside, 0·488 mg., there is a linear relation between the extinction at 565 mμ. and the amount of added fructose over the range 2–5 mg. fructose. The volume of the solution was 12 ml., 1 ml. aqueous solution of

the glucoside and fructose and 11 ml. reagent *A*. The value of the extinction at 565 mμ., which is the peak of the characteristic band of the red pigment, measured in a Hilger-Nutting Visual Spectrophotometer, are recorded in Table 3.

Table 3. *Relation between fructose concentration and extinction at* 565 mμ.

Fructose (mg./12 ml. solution)	Extinction at 565 mμ.
1·97	1·12
2·97	1·22
4·94	1·45

Fructose (2 mg.) in a final volume of 12 ml. gives a strong colour. Brominated asperuloside gives hardly any colour with reagent *A* unless fructose is added. So that if this compound is used and the volumes are proportionately reduced to 1–2 ml. total the reaction may prove to be of use for quantitative work down to 50 μg. ketohexose.

The effect of tryptophan and other heterocyclic nitrogen compounds. Tryptophan behaves like a ketohexose in changing the colour from blue towards red, in the case of asperuloside, aucubin, hexa-acetyl aucubin and bromo-hexa-acetyl aucubin, and in producing a red-purple colour with brominated asperuloside. No other amino-acid has a marked effect, although cysteine intensifies band I of the asperuloside colour. Combined tryptophan reacts like free tryptophan. With asperuloside and tryptophan the purple colour develops rapidly at 70°, whereas with ketose the temperature must be raised to 100° before the reaction will occur readily.

Pyrrole compounds produce a similar effect. With the ethyl ester of 3:5-dimethylpyrrole-2-carboxylic acid asperuloside gives a bright magenta pigment with strong absorption between 500 and 550 mμ. Aucubin and its derivatives give intense purple colours. Carbazole, which gives colour reactions with the acid degradation products of sugars, gives a purple colour with asperuloside and a slight reddening of the colour with aucubin.

The effect of some phenolic substances. Some phenolic substances give a reddening of the colour with the two glucosides and their derivatives. Phenol, cresol, orcinol, resorcinol, pyrogallol, thymol, β-naphthol, syringin and phlorrhizin react in this way. There is no effect with quinol, salicylic acid and catechol. Phloroglucinol gives a red-brown colour with asperuloside and green with aucubin. Brilliant red-purple colours are obtained with orcinol, resorcinol and thymol.

Other colour reactions of aucubin and asperuloside. Since a furan structure has been proposed for aucubin, the reactions of the two glucosides with known colour reagents for furan derivatives were studied. These reactions are the $SbCl_3$ reaction (Wettstein & Miescher, 1943), the reaction with Ehrlich's aldehyde reagent (Reichstein, 1932), the reaction with vanillin and HCl (Asahina, 1924–6) and Shear's reaction with aniline hydrochloride (Levine, Seaman & Shaughnessy, 1933).

Antimony trichloride reaction. Karrer & Schmid (1946) found that $SbCl_3$ reacts with aucubin to give a strong blue-violet colour in the cold. To a few mg. of solid glucoside 0·1 ml. acetic anhydride and 0·4 ml. of a saturated solution of $SbCl_3$ in pure $CHCl_3$ containing 1% (v/v) ethanol were added. Controls without $SbCl_3$ remained colourless for 24 hr. The development of colour from aucubin and asperuloside in the cold is described in Table 4. If the solutions are warmed the colours develop rapidly.

Table 4. *The rate of development of colour from asperuloside and aucubin in the antimony trichloride reaction*

Time (min.)	Colour: Aucubin	Colour: Asperuloside
0	Pale yellow	—
2	Pale green	—
7	Pale blue	—
10	Pale blue-violet	Pale green
30	Strong blue-violet	Blue-green

Action of Ehrlich's reagent and vanillin in conc. HCl. The glucosides were tested with Ehrlich's aldehyde reagent under the strongly acid conditions described by Karrer & Schmid (1946). Asperuloside produces the same blue colour as aucubin but more slowly (Table 5). Both colours are stable.

Asahina (1924–6) showed that when substituted furan compounds such as furfurals are treated with a saturated solution of vanillin in cold conc. HCl, colours develop. Aucubin rapidly gives a red colour and asperuloside gives a magenta colour more slowly.

The reactions with Ehrlich's reagent and with vanillin show a similarity. By analogy with pyrrols (Fischer & Orth, 1934), it may be suggested that the aldehyde group of the reagent condenses with two molecules of the furan compound to produce a substance analogous to the triphenylmethane type which is oxidized to give coloured products.

Reaction with aniline hydrochloride. Furan compounds react with aniline salts to produce coloured derivatives such as the well known red colour formed by the action of aniline acetate on furfural. Asperuloside reacts in the cold with Shear's reagent (1 vol. of conc. HCl dissolved in 15 vol. of

Table 5. *Rate of development of colour from aucubin and asperuloside with Ehrlich's reagent*

Time of heating to 75° ...	Colour: 0 sec.	30 sec.	60 sec.	90 sec.	4 min.	10 min.
Glucoside						
Aucubin	Yellow	Blue-violet	Blue-violet	Blue-violet	Blue-violet	Blue-violet
Control, no Ehrlich reagent	—	Faint green	Brown	Brown	Brown	Brown
Asperuloside	Yellow	Yellow	Yellow	Yellow	Faint green	Blue-violet
Control	—	—	—	—	Faint brown	Brown

aniline). It gives a yellow, brown and finally an intense green colour. Aucubin does not react in the cold; on heating to 100° for a short time it gives a brown colour indistinguishable from that obtained with glucose under the same conditions.

Reaction of asperuloside with amines. Reactions with amines under neutral or faintly acid conditions sharply distinguish asperuloside from aucubin, which gives no colour reactions with amines. Asperuloside reacts with primary amines to form strongly coloured products. A few mg. each of amine and asperuloside heated in an open dish on a boiling-water bath with a few drops of 10% (v/v) acetic acid, 2–3 ml. water and an excess of sodium acetate produce a strong colour, usually within 10 min. In most cases the pigment is soluble in water, insoluble in organic solvents, and blue or blue-violet in colour. With methylamine the reaction is very rapid, and a strong colour develops in 1 or 2 min. This pigment is blue-violet with a marked absorption band from 530 to 610 mμ. It will also develop on standing at room temperature. It is insoluble in amyl alcohol. Octadecylamine reacts much more slowly and heat is required. The blue pigment produced is soluble in amyl alcohol, which suggests that these blue substances are condensation products of the whole amine molecule with the whole or part of the asperuloside molecule.

All the naturally occurring amino-acids, except cysteine, and a number of di- and tri-peptides react with asperuloside, and in all cases the pigment is blue or blue-violet with a characteristic absorption band similar to that obtained with methylamine. The proteins wheat gluten and gelatin reacted to give blue-violet colours. Cysteine and pyrrolidonecarboxylic acid do not react to give coloured products, but cysteic acid gives the usual colour.

Some amino compounds are either non-chromogenic with asperuloside or do not give the typical reaction, which appears to require a free amino group. Secondary and tertiary amines do not react as a rule, and while L-leucine behaves like the other α-amino acids acetyl L-leucine gives no colour. Amides such as succinamide, acetamide and urea are not chromogenic but formamide produces the blue-violet colour. Hydroxylamine and Ehrlich's aldehyde reagent are not chromogenic with asperuloside, but aniline gives a strong green-blue colour. Heated under the mildly acid or neutral conditions of this test ammonia produces a soluble blue-violet colour and a blue-violet precipitate, but under other conditions a different reaction occurs. If asperuloside is allowed to stand with ammonium acetate in the cold a deep-brown colour gradually develops. Dimethylamine reacts rapidly in the cold to produce a brown pigment, *o*-phenylenediamine gives a black precipitate on heating, hydrazine a strong red-brown and phenylhydrazine an olive-green. Glucosamine gives first a violet colour and then a strong brick-red with two absorption bands in the visible spectrum, one at 540–560 mμ., maximum at 550 mμ. and a weaker but sharper band at 600 mμ.

The substances which give characteristic red pigments with asperuloside when heated with reagent *A* give no coloured products under these faintly acid or neutral conditions. Naturally tryptophan is an exception and gives a typical amine reaction.

These reactions with amines do not seem to be due to the presence of a furan nucleus in asperuloside, but rather to the presence of carbonyl groups or potential carbonyl groups, and may be compared with the purple colour reactions of ninhydrin with amino-acids. This interpretation is supported by the fact that, after standing with N/10-NaOH for 5 min., asperuloside no longer gives the amine colour reaction, whereas those reactions which can be attributed to a furan structure are unaffected. Further support is lent to this view by the fact that aucubin gives no colour reactions with primary aliphatic amines.

Action of carbonyl reagents. Since the above reactions suggest that asperuloside may contain or generate carbonyl groups, further tests were made. The Tollens silver nitrate test and the action of Schiff's reagent suggest that there is no aldehyde group. It has already been shown that asperuloside reacts with *o*-phenylenediamine and hydrazine. It also reacts with hydroxylamine hydrochloride in acid solution in the cold, as may be shown by Duke's (1944) test. The reagent is made by dissolving 0·5 g. hydroxylamine hydrochloride in 95% ethanol containing bromophenol blue and adjusting the pH to 3·7–3·9. If an aldehyde or ketone is added to this solution hydroxylamine combines with the carbonyl groups and an equivalent amount of HCl is released. Titration back to pH 3·7–3·9 gives an estimate of the carbonyl radical. Asperuloside (46·7 mg.) was dissolved in 3 ml. of the solution and allowed to stand at room temperature. At intervals the solution was titrated to pH 3·8 with N/70-NaOH. The figures in Table 6 suggest that carbonyl groups are slowly formed.

Table 6. *The reaction of asperuloside with hydroxylamine hydrochloride*

(Results expressed as ml. N/70-NaOH required to neutralize the HCl liberated. 7·8 ml. ≡ 1 carbonyl group per mol. At all times the control without NH_2OH showed no detectable free acid.)

Time (hr.)	N/70-NaOH (ml.)
15	2·05
17	2·65
18	3·17
39	5·52
63	6·77

Asperuloside acetate and aucubin do not react with hydroxylamine under these conditions. These results are in keeping with the presence of at least one potential carbonyl group in the asperuloside molecule, but the experiment is not conclusive for the glucoside undergoes chemical changes under mild conditions. The reactions with other carbonyl reagents are also inconclusive, and an adequate interpretation of these observations awaits a more rigorous chemical study.

Other glycosides similar to aucubin and asperuloside

Aucubin has been reported in many plants besides those from which it has been isolated. Extracts of some plants, particularly members of the family Scrophulariaceae, produce reducing sugar and a dark amorphous precipitate when treated with emulsin. This behaviour is characteristic of true aucubin-containing plants and is shared by those containing asperuloside. By means of the tests described in this paper it has been shown that while some of these glucosides are similar to aucubin they are not identical with it. In addition a very characteristic test with phloroglucinol and HCl, developed by one of us (R.H.) in collaboration with Mr J. L. Crosby, indicates this difference in a striking

manner and shows that similar glycosides occur in the family Labiatae.

The phloroglucinol blue reaction. If a few ml. of an extract of the tubers of *Stachys palustris* (Labiatae) in 50% ethanol is mixed with a few mg. of phloroglucinol and an equal volume of concentrated HCl in the cold, an intense, unstable, green-blue colour develops in a few seconds. These conditions are the same as for the well known phloroglucinol-HCl test for lignin. On dilution with water the colour becomes bright blue and may be extracted quantitatively with butanol or amyl alcohol. If the acid is removed by repeated washing with water the colour becomes rich purple. It is then more stable and may be extracted into alkaline aqueous solutions. With the exception of naphthoresorcinol no other phenolic compound has been found to give such a distinctive colour reaction with the glucoside. Extracts from some other Labiatae: *S. tuberifera*, *S. sylvatica*, *Teucrium scorodonia*, *Ajuga reptans* and *A. chamaepitys* give a similar reaction. These chromogenic substances appear to be glycosides, and unsuccessful attempts were made to isolate them from *Stachys palustris* and *Ajuga reptans*. They were readily adsorbed by charcoal and eluted by aqueous ethanol.

The phloroglucinol blue reaction is given by extracts of some members of the family Scrophulariaceae: *Antirrhinum major*, *Linaria vulgaris*, *Linaria cymbalaria*, *Verbascum thapsus* (and cultivated varieties) and *Scrophularia nodosa*. In these cases the reaction was slower and the first blue colour was of a greener hue, but on dilution with water it became bright blue. Unlike the substances from the Labiatae those from *Antirrhinum major* and *Linaria vulgaris* are stable and may be obtained in a crude amorphous form by the charcoal adsorption method. Filter-paper chromatography using butanol, acetic acid and water or butanol, pyridine and water systems showed that both *Linaria* and *Antirrhinum* contain two components giving identical phloroglucinol-HCl reactions.

Besides the very distinctive colour reactions with phloroglucinol or naphthoresorcinol and HCl all these substances give some of the colour reactions characteristic of aucubin and asperuloside. Some examples are given in Table 7.

Both aucubin and asperuloside react in the phloroglucinol-HCl test, but the colour produced is not blue; aucubin gives a purple colour rapidly, the control without phloroglucinol is brown; and asperuloside gives a purple colour, the control without phloroglucinol is grey-green.

Hitherto asperuloside has been thought to be a specific product of plants in the family Rubiaceae. However, as has been described earlier in this paper, it also occurs in *Daphniphyllum macropodum*, a Chinese plant of uncertain affinity, classified in the family Euphorbiaceae. Also, using the specific colour tests for asperuloside, which showed the presence of asperuloside in *D. macropodum*, two more closely allied glycosides have been detected. One of them, occurring in the uncommon saprophytic plant *Monotropa hypopitys* Walt. is the glucoside monotropéine isolated by Bridel (1923). *Monotropa* is classified in Ericales, family Pyrolaceae. The other glycoside occurs in *Genipa americana* (family Rubiaceae). There are large quantities of it in the pulp of the unripe fruit.

Both glycosides may be partially purified by the charcoal adsorption method. Their solutions give a blue colour on heating with reagent *A* and the spectrum has two characteristic absorption bands in the visible range, a strong band I at 630–640 mμ. and a weaker band II at 600 mμ. If heating is continued the colour becomes purple and a third band III appears at 550–570 mμ. It is unlikely that this is due to contamination with ketose or other substances known to give a purple reaction with asperuloside and reagent *A*, because these characteristic reddening reactions are all given on brief heating of the two glycosides with ketoses, heterocyclic N compounds or phenols and reagent *A*.

Both glycosides give the blue-violet reaction with primary amines which is characteristic for asperuloside. Bridel (1923) states that monotropéine is hydrolysed by emulsin to a blue product. We found that both monotropéine and asperuloside give a blue-violet, water-soluble pigment on incubation at 37° for about 1 hr. with crude emulsin and concluded that the colour is due to the reaction of the glycosides with primary amino groups in the enzyme preparation and does not necessarily depend upon hydrolysis of the glycosidic linkage, for the intensity of the colour varied very greatly with different samples of emulsin and, in the case of asperuloside, occurred after heat inactivation of the enzyme. In support of this it was found that the reaction between asperuloside and glycine to form the blue-violet pigment occurs at 37° in neutral solutions, and is appreciable after less than 30 min. incubation. Under these conditions monotropéine does not react unless emulsin is added; if glycine is also added the rate of colour development is greatly increased.

Our interest in *Genipa americana* was aroused by the description of the dyeing properties of the fruit given by Bancroft (1813). We suggest that the blue or purple colours observed to develop in the bruised tissues and extracts of *Genipa* fruit are due to a reaction between the asperuloside-like glycoside and some aliphatic amino compound also in the plant.

Filter-paper chromatography shows that both monotropéine and the glycoside from *Genipa* are more water-soluble than asperuloside and the ratio of the R_F values of

Table 7. *Colour reactions of glycosides from* Antirrhinum, Ajuga *and* Stachys

Source of glycoside	Colour developed		
	Antirrhinum major	*Ajuga reptans*	*Stachys palustris*
Test			
Phloroglucinol-HCl	Blue	Blue	Blue
Reagent *A*	Green-brown	Purple to blue	Violet to green
Reagent *A* and fructose	Red-brown	Brown to purple	Brown
Reagent *A* and fructose, glucoside previously treated with Br_2	—	Bright red	Brown-violet
Ehrlich's reagent	Green	Blue	Blue
Antimony trichloride	Violet	Brick red	Brown-violet
Shear's reagent in the cold	Red-brown	Brown	Bright red

these compounds and asperuloside run in butanol-acetic acid-water system (Partridge & Westall, 1948) is 1·6:1.

Bridel (1923) showed that monotropéine is a monoglucoside and contains an acidic group.

DISCUSSION

The glycosides aucubin and asperuloside are both derived from aglycones which cannot be isolated in the free state. They give dark resinous products when the glucose is removed, either by mild acid hydrolysis or by the action of hydrolytic enzymes derived from plant sources. It has now been found that if the acid hydrolysis is carried out in the presence of oxygen and a catalyst (reagent *A*) definite coloured products are obtained, which show characteristic absorption spectra.

A variety of reactions given by aucubin and asperuloside have been compared; and it has been concluded that asperuloside has similarities to aucubin which would be explained if the aglycone of asperuloside had a substituted furan structure like that ascribed to the aglycone of aucubin by Karrer & Schmid (1946). Asperuloside is a more reactive and, in certain respects, less stable glycoside than aucubin, as may be seen from the comparison in Table 8.

If the 'aucubin type' of glycosides can be accepted, it is clear that the group is of very wide distribution, and must therefore represent a fairly common feature of plant metabolism. Aucubin itself has already been isolated from members of seven families of plants, and its isolation from *Buddleia*, described here, has added an eighth. The occurrence of aucubin in *Eucommia ulmoides*, described by Plouvier (1944), is of both taxonomic and metabolic interest. This plant is difficult to classify and does not appear to be related to the families already known to produce aucubin.

The characterization of asperuloside as a probable representative of the 'aucubin type' of glycosides extends the taxonomic range of the class; and its isolation from *Daphniphyllum macropodum* (Euphorbiaceae) shows that this glycoside is not a specific product of the Rubiaceae. The detection of a glycoside akin to asperuloside in *Genipa americana* (Rubiaceae) is the first indication that other substances related to asperuloside may be produced by the Rubiaceae. Further, the characterization of 'monotropéine', a product of *Monotropa hypopitys* Walt., placed in the Ericales, family Pyrolaceae, now makes it reasonable to believe that asperuloside and very closely related glycosides may be of wide occurrence.

Table 8. *Summary of the chemical characteristics of aucubin and asperuloside*

	Aucubin	Asperuloside	Remarks
Empirical formula	$C_{16}H_{22}O_9$	$C_{17}H_{24}O_{11}$	Asperuloside has 2-O and 2-H atoms more than aucubin
Acetate	$C_{16}H_{16}O_9(COCH_3)_6$	$C_{17}H_{16}O_{11}(COCH_3)_8$	Asperuloside has 2-OH groups more than aucubin
Acetate + Br_2	Substitution	No substitution	No free —CH= in proposed furan nucleus of asperuloside
Reagent *A*	Blue	Blue	—
Furan reagents	+	+	Asperuloside reacts more slowly
Action of primary amino compounds	None	+ (negative after alkali)	Presence of potential carbonyl group or groups in asperuloside
Action of carbonyl reagents	None	+	
Stability to OH^-	Stable	Rapidly modified, acid liberated, no amine reaction; reaction with reagent *A* unaffected	Lactone group may be present in asperuloside

The colour reactions have been used to test glycoside preparations from a wide variety of plants, some of which have been said to contain aucubin. These reactions provide evidence for the definition of a class of glycosides of the 'aucubin type' showing a number of individual variations of structure (see Table 7). So far aucubin is the only member of this class on which sufficient data have been obtained to justify a constitutional formulation; and even some of the readily accessible plant sources, containing considerable amounts of glycosides of this class, have not, so far, yielded crystalline products.

The three glycosides discussed in the previous paragraph are sharply distinguished from the other glycosides of the 'aucubin type' by their ready reaction with primary amino compounds giving a purple colour. It is clear that this reaction, which is analogous to the reaction of ninhydrin with amino-acids, does not involve a furan nucleus. The capacity of the glycosides to give this reaction is destroyed by brief treatment with mild alkali, whereas the furan-like reactions with acid reagents are unaffected, even by much more drastic alkaline treatment.

The reaction with phloroglucinol and hydrochloric acid, together with some of the other reactions described in this paper, show that the 'aucubin type' of glycoside also occurs in the Labiatae. The aucubin-like glycosides which give the phloroglucinol-hydrochloric acid reaction are found particularly in the Scrophulariaceae, where several structural variants occur. Aucubin itself may be isolated from *Melampyrum* (Braecke, 1923*a*), *Veronica hederaefolia* (Charaux, 1922) and *Rhinanthus* (Braecke, 1923*b*), all Scrophulariaceae. Glycosides giving the phloroglucinol-hydrochloric acid reaction were found in the genera *Stachys* and *Ajuga* in the family Labiatae.

The 'aucubin type' of glycosides is thus characterized by the immediate formation of coloured, resinous secondary bodies on acid hydrolysis of the colourless glycoside and by reactions similar to substituted furan compounds. It cannot be suggested that the furan ring itself is necessarily present in all glycosides of this class, for other types of compounds give some of the reactions of substituted furans. Terpenes like geraniol give colour reactions with phloroglucinol and hydrochloric acid in the cold, and pyruvic acid gives one on heating. The antimony trichloride reaction (Levine & Richman, 1933) and the aniline hydrochloride reaction (Levine *et al.* 1933) are given by some terpenes. In certain cases the two reactions occur readily in the cold, the ease of reaction being increased by unsaturation and the presence of carbonyl groups.

The colour reactions given by the 'aucubin type' of glycosides provide a technique for the study of these substances in relation to the living plant. The only detailed chemical study of these glycosides is that of aucubin by Karrer & Schmid (1946).

SUMMARY

1. The method of Hill & Van Heyningen (1951) has been applied to the isolation of aucubin and asperuloside from plants.

2. Aucubin has been isolated for the first time from *Buddleia globosa* (family Loganiaceae), bringing the number of families known to produce aucubin to eight.

3. Asperuloside has been isolated for the first time from a non-rubiaceous plant, *Daphniphyllum macropodum* (Euphorbiaceae).

4. The empirical formula $C_{17}H_{24}O_{11}$ has been suggested for asperuloside.

5. A crystalline acetate of asperuloside has been obtained.

6. Aucubin, asperuloside and some of their derivatives are extremely acid-labile and under specified acidic conditions produce blue colours. In the presence of some phenols, ketoses and heterocyclic nitrogen compounds characteristic red colours are produced in this reaction.

7. These colour reactions and certain others are shared by other substances, which appear to be glycosides and which occur in the families Scrophulariaceae, Labiatae, Pyrolaceae and Rubiaceae.

8. It has been proposed on the basis of the work of Karrer & Schmid (1946) that all these glycosides could be regarded as substituted furan derivatives like aucubin.

9. The existence of a class of glycosides, typified by aucubin, is postulated. These glycosides are produced by a wide variety of plants.

We are indebted to Dr A. C. Chibnall, F.R.S., and Dr C. S. Hanes, F.R.S., for their interest in this work, and also to Mr E. J. H. Corner for advice and invaluable help in obtaining some of the plant material we have used. Leaves of *Daphniphyllum macropodum* were provided by Mr J. Gilmour, Director of the Royal Horticultural Society's Garden, Wisley, and samples of ketose derivatives were the gift of Dr W. A. Johnson of the Chemical Laboratory, Cambridge. We are grateful to Dr D. J. Bell for a sample of D-tagatose, Dr J. Needham, F.R.S., for L-sorbose and Mr F. J. R. Hird for samples of peptides used in this work. The work has been carried out as part of a programme of the Agricultural Research Council's Unit of Plant Biochemistry, Cambridge.

REFERENCES

Armstrong, E. F. & Armstrong, K. F. (1931). *The Glycosides, Monographs on Biochemistry*, p. 21. London: Longmans Green.

Asahina, Y. (1924–6). *Acta phytochim., Tokyo*, **2**, 1.

Bancroft, E. (1813). *Experimental Researches Concerning the Philosophy of Permanent Colours and the best Means of Producing them, by Dyeing, Calico Printing &c.* **1**, 260. London: Cadel and Davies.

Bergmann, M. & Michalis, G. (1927). *Ber. dtsch. chem. Ges.* **60**, 935.

Bourdier, L. (1907). *J. Pharm. Chim., Paris*, (6), **26**, 254.

Bourquelot, E. & Hérissey, H. (1905). *Ann. Chim. (Phys.)* (8), **4**, 289.

Braecke, M. (1923*a*). *Bull. Soc. Chim. biol., Paris*, **5**, 207.

Braecke, M. (1923*b*). *Bull. Soc. Chim. biol., Paris*, **5**, 258.

Bridel, M. (1923). *Bull. Soc. Chim. biol., Paris*, **5**, 722.

Bridel, M. (1929). *Bull. Soc. Chim. biol., Paris*, **11**, 620.

Charaux, C. (1922). *Bull. Soc. Chim. biol., Paris*, **4**, 568.

Duke, F. R. (1944). *Industr. Engng Chem.* (Anal. ed.), **16**, 110.

Fischer, H. & Orth, H. (1934). *Die Chemie des Pyrrols*, Vol. 1, 67. Leipzig: Akademische Verlagsgesellschaft.

Hérissey, H. (1925*a*). *Bull. Soc. Chim. biol., Paris*, **7**, 1009.

Hérissey, H. (1925*b*). *J. Pharm. Chim., Paris*, (8), **2**, 177.

Hérissey, H. (1926*a*). *J. Pharm. Chim., Paris*, (8), **3**, 353.

Hérissey, H. (1926*b*). *J. Pharm. Chim., Paris*, (8), **4**, 481.

Hérissey, H. (1926*c*). *Bull. Soc. Chim. biol., Paris*, **8**, 489.

Hérissey, H. (1927). *Bull. Soc. Chim. biol., Paris*, **9**, 953.

Hérissey, H. (1933). *Bull. Soc. Chim. biol., Paris*, **15**, 793.

Hérissey, H. & Lebas, C. (1910). *J. Pharm. Chim., Paris*, (7), **2**, 490.
Hill, R. & Van Heyningen, R. (1951). *Biochem. J.* **49**, 332.
Karrer, P. & Schmid, H. (1946). *Helv. chim. Acta*, **29**, 525.
Levine, V. E. & Richman, E. (1933). *Biochem. J.* **27**, 2051.
Levine, V. E., Seaman, C. le R. & Shaughnessy, E. J. (1933). *Biochem. J.* **27**, 2047.
Partridge, S. M. & Westall, R. G. (1948). *Biochem. J.* **42**, 238.
Plouvier, V. (1944). *C.R. Acad. Sci., Paris*, **218**, 605.
Reichstein, T. (1932). *Helv. chim. Acta*, **15**, 1110.
Wettstein, A. & Miescher, K. (1943). *Helv. chim. Acta*, **26**, 788.
Zellner, J. (1934). *Arch. Pharm., Berl.*, **272**, 601.

The Accumulation and Utilization of Asperuloside in the Rubiaceae

By A. R. TRIM
Biochemical Laboratory, University of Cambridge

(*Received* 14 *February* 1951)

The significance of glycosides in plant metabolism is obscure, and the present work has been undertaken as a contribution to its elucidation. Large amounts of glycosides are present in many plant cells and although these substances vary widely in their chemical constitution many of them exhibit a common pattern of distribution in the plant. They are accumulated in the vacuoles of young parts of plants during the period of cell expansion, and afterwards may be partly or completely removed as the part ages.

The plants investigated were: *Galium aparine* (cleavers, or goose-grass), *Asperula odorata* (woodruff) and *Rubia tinctorum* (dyer's madder), which belong to the tribe Stellatae of the family Rubiaceae. Most plants produce glycosides of a number of aglycone types, and the Stellatae are known to produce anthocyanins and related glycosides, which are universal among higher plants, anthraquinone glycosides, coumarin glycosides and asperuloside, which probably has a substituted furan structure (Trim & Hill, 1952).

Asperuloside was chosen for study because it occurs in very large quantities in the plants investigated, and because its chromogenic properties provide very simple means for its detection and estimation.

EXPERIMENTAL

The methods of growing and collecting the different plant materials will be described with the experiments in which they were used. The time of collecting was mid-morning. Extracts were made from fresh, weighed material unless otherwise stated. Dry weights were determined after drying equivalent batches of the material for several days at 37° and then over concentrated H_2SO_4 and solid NaOH *in vacuo*. The parts to be extracted were ground with an equal weight of 0·1 N-HCl and a little sand. A few drops of $CHCl_3$ were added and the mush allowed to stand for about 15 min. The ground tissues were then mixed with kieselguhr, filtered on a Büchner funnel and washed with water until the runnings were free from asperuloside. The extract was made up to an appropriate volume with water, neutralized with $CaCO_3$ and filtered.

Estimation of asperuloside. 1 ml. samples of the extracts were mixed with 11 ml. of reagent *A* (Trim & Hill, 1952) and heated on a boiling-water bath for 5 min. with occasional shaking. The characteristic blue derivative of asperuloside developed. The cooled solution was compared with a standard in a Duboscq-type visual colorimeter. Beer's Law applies over a wide range of concentrations as may be seen in Table 1. The effect of the addition of a plant extract is shown in Table 2. The colour developed under these conditions has an absorption spectrum with bands at 645 and 600 mμ. Early in the season, when most parts of the plants were young, very little extraneous colour was developed. Later, when the bulk of the tissues were older, the plants produced sufficient quantities of interfering substances to necessitate the use of a red filter when making the colorimetric measurements. The nature of the interference by substances such as phenols, ketoses and heterocyclic N compounds, which are likely to occur in plants, has been

Table 1. *Colorimetric estimation of known amounts of asperuloside*

Asperuloside (mg.)	Recovery, using a 1 mg. standard (%)
0·25	108
0·50	100
0·75	100
1·0	100

Table 2. *Colorimetric estimation of known amounts of asperuloside in the presence of an extract of* Galium aparine

Extract (ml.)	Asperuloside added as 0·1% solution (mg.)	Total found (mg.)	Recovered (mg.)
0·5	0·0	0·21	0·0
0·5	0·10	0·31	0·10
0·5	0·25	0·46	0·25
0·5	0·50	0·71	0·50
0·0	0·50	0·50	0·50

described (Trim & Hill, 1952). Since relatively large proportions of these substances must be added to cause a marked effect, the interference was rarely serious.

Reagent *A* was also used for the detection of asperuloside in portions of plant tissues. The blue colour develops in sections and fragments of plant heated with reagent *A* on a slide or in a test tube. This procedure was used to detect the localization of asperuloside.

Galium aparine

The material was collected at random from one large plot of plants. Ripe seeds were collected from the plot at the end of the season 1946 and used for experiments during the following winter and spring.

Seeds and seedlings. Batches of 30 newly ripened seeds were analysed and found to contain 3·5% asperuloside on a dry-weight basis. After storing until the following spring the value had fallen to 2·5% and the seeds remained viable. Qualitative tests on sections which had been cut from seeds soaked in water for 16 hr. showed that the endosperm and the whole of the embryo contained asperuloside. As germination proceeds the endosperm shrinks and the amount of asperuloside in it declines. The embryo develops at the expense of the endosperm. Asperuloside was found in all parts of the developing embryo and was in particularly high concentrations in the early embryonic hypocotyl and cotyledons. These parts gave a purple colour with reagent *A*, which suggested the presence of a large proportion of the interfering substances mentioned above. The interfering material disappeared during the development of the seedling. As the seedlings grew they were dissected into their parts and each part was tested separately. This confirmed the observation that asperuloside occurs in all parts of the very young seedling and that there is a high concentration in the cotyledons. The glycoside appears to be absent from the growing tips of the roots.

The following measurements show how asperuloside accumulates during the period of germination. Two batches of about 500 seeds were sown on wet sand in two shallow dishes covered with sheets of glass, slightly raised above the tops of the dishes to admit air. Both dishes were kept in a greenhouse at 18° and one of them was covered to exclude light. At intervals two samples of 30 seeds were removed from each dish and ground with sand and a few ml. 3% HCl to extract the asperuloside. This was estimated, and the values obtained are plotted in Fig. 1. The initial fall in the amount of asperuloside per individual takes place while the sprouting radicle has still not emerged through the seed coat. By the time the minimum has been reached sprouting is just visible among the seeds which have been kept in the dark. These seedlings then accumulate asperuloside until the amount per individual reaches the level in the ungerminated seed, and if the seedlings are kept dark this level is maintained for many days. By the time the maximum has been reached the plant has fully emerged. Exposure of these seedlings to the light results in an immediate marked increase of asperuloside, due presumably to photosynthesis. The sprouting of the seedlings grown in the light is retarded by about 8 days and the accumulation of asperuloside is slower, but by the time the cotyledons appear the amount of asperuloside has reached the level of the maximum for seedlings grown in the dark. There is then a rapid increase in the amount of asperuloside due to the supervention of photosynthesis.

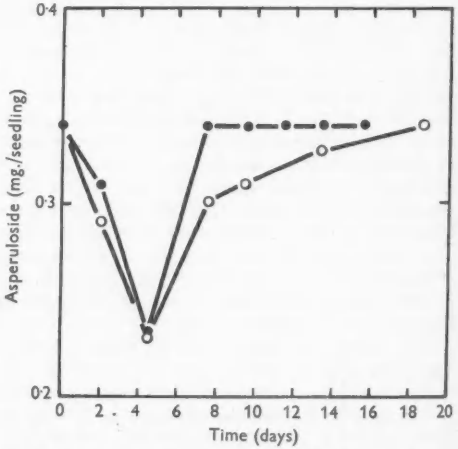


Fig. 1. The production of asperuloside in germinating seeds of *G. aparine*: (i) ●—●, seeds germinated in the dark; (ii) ○—○, seeds germinated in the light.

This experiment was repeated a month later and the same changes were observed. The amount of asperuloside in the seedlings germinated in the dark reached 0·34 mg. per plant after 12 days and remained constant for another 10 days. Then it fell off slowly, reaching 0·26 mg. per plant 32 days from the initiation of germination. Some of the seedlings germinated in the dark were exposed to the light 18 days after the beginning of the experiment and immediately began to produce more asperuloside. After 2 days' exposure there was 0·4 mg. per plant and after 15 days 0·59 mg. per plant.

It is concluded from these experiments that asperuloside is continuously accumulated by the germinating embryo in the absence of external nutrients other than water, the initial rapid fall in the amount of glycoside being due to the utilization of the endosperm. This accumulation is terminated abruptly at a certain level unless photosynthesis is permitted. The effect of feeding through the roots was not tested.

Asperuloside formation in seedlings grown in soil. Twelve 25 cm. pots of soil were each sown with 40 seeds of *G. aparine* and kept in a greenhouse at 18°. After a month, when the seedlings were well developed, they were carefully removed from two of the pots, washed, counted and each plant divided into four parts: roots, hypocotyl, cotyledons and shoots. The parts from one pot were weighed and the asperuloside was extracted. The parts from the other pot were used for the determination of the dry weights. Further samples were taken as the seedlings developed. Some of the results are plotted in Fig. 2.

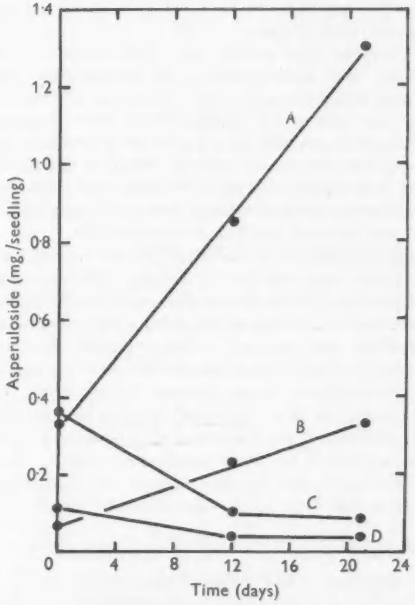


Fig. 2. The accumulation of asperuloside in seedlings of *G. aparine* grown in soil with normal lighting; (*A*) leaves, (*B*) roots, (*C*) cotyledons, (*D*) hypocotyl.

As the plant develops there is continuous steady accumulation of asperuloside in the roots and shoots. At the same time, the amount in the cotyledons falls to a constant low level. While there is a possibility of transport of glycoside from the organs which are losing it to those which are gaining it, at least part of it is synthesized anew and accumulates in the growing parts, for the amount of glycoside which is gained by the roots and shoots separately is greater than the sum of the amounts lost by the hypocotyl and cotyledons over the period of the experiment. Figures for dry weights (Table 3) show that in the course of this experiment the cotyledons and hypocotyl were no longer growing, while the shoots and roots were rapidly increasing in weight.

Table 3. *Dry weights of parts of* Galium aparine *seedlings*

Day	Dry weight (mg.) Hypocotyl	Cotyledons	Shoots	Roots
0	3·3	6·6	7·4	17·0
12	2·6	5·2	27·0	23·0
21	1·2	5·2	58·0	58·0

The seasonal variation of the amounts of asperuloside. Individual shoots from different plants at the same stage of growth varied considerably in their asperuloside content. It was found that significant values for comparative purposes could be obtained by analysing batches of six or more shoots picked at random from the experimental plot. The values for six batches of six shoots picked at the same time are tabulated in Table 4.

Table 4. *Asperuloside in the shoots of* Galium aparine

(Batches of six shoots.)

Batch no.	Wet wt. (g.)	Asperuloside (% of dry wt.)
1	8·9	4·8
2	10·7	4·8
3	10·9	4·8
4	11·9	5·3
5	8·2	4·8
6	6·4	5·3

The formation of the glycoside throughout the season was followed. Batches of 20 shoots were taken for as long as the rapidly increasing size of the shoots permitted. After that the number was reduced. The amount of glycoside per shoot gradually rises and then falls sharply as the shoot becomes senescent. When different parts of the shoot were tested with reagent *A* it was found that asperuloside is present in large amounts in all parts of the very young leaf, with the possible exception of the epidermis and chlorenchyma. There is much less in old leaves and what they do contain is confined to the vascular tissues, showing that the decline, already observed in the ageing hypocotyl and cotyledons, also occurs in the leaves.

The manner in which the glycoside content of the plant changes with age was shown more clearly by the following experiments with growing shoots, which were cut up into successive nodes, defined as follows. The leaves grow in whorls about the nodes. Each node and the internode above it develop together so that their parts have the same average age and may be taken as a unit composed of tissues of approximately the same age. Dry weights were determined on material from a similar shoot from

the same plant and a corresponding node. The results in Fig. 3 show that where rapid, exponential growth is taking place there is a high, constant concentration of asperuloside; after exponential growth has ceased there is a sharp downward trend in asperuloside concentration towards a lower, slowly drifting level.

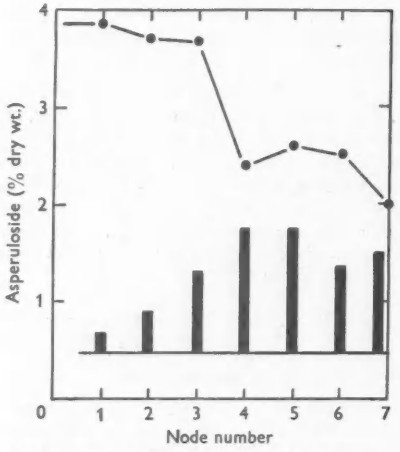


Fig. 3. The concentration of asperuloside in successive nodes of a shoot of *G. aparine*. Node 1 is the most apical. The black columns indicate the relative dry weights of the nodes.

If the change in asperuloside concentration is considered in relation to the development of an individual mesophyll cell the change from the predominance of accumulation to the predominance of utilization (or transport away from the leaf) appears to be sharp and to coincide with the end of vacuolation and cell expansion. In this case (and all the others described in this paper) the change does not occur in a particular node, say 3 or 4, counting from the apex of the shoot downwards, but appears to be at a hypothetical point between node 3 and 4 (in this case). This is probably to be explained by the fact that all the cells in a particular node are not at the same stage of development. In this case all the cells in node 4 have passed through the stage of exponential growth; but while some of those in node 3 have also passed through that stage many of them are still in it. In this situation it is logical to expect the asperuloside concentration in node 3 to be on the higher level, although growth has already begun to slow down.

It seemed that during the stage of exponential growth the accumulation of asperuloside was directly proportional to the increase in dry matter. This was tested by estimation of asperuloside in shoots of *G. aparine* over a period of 2 weeks. The shoots were chosen so that the whole of the tissues were well within the stage of exponential growth. They were picked at random from the experimental plot and divided into three batches of 30.

Asperuloside was estimated in two batches and the third was used for dry-weight determinations. The results in Table 5 show that over a period during which the bulk of the average shoot increased nearly threefold the amount of asperuloside remained directly proportional to the fresh and dry weights.

Accumulation of asperuloside in the fruit. The accumulation of the glycoside during the period of the swelling of the fruit was also observed. Fruit was collected at three successive stages. As is shown in Table 6, the amount of glucoside is approximately proportional to the dry weight.

Table 5. *Proportion of asperuloside in the shoots of* Galium aparine *in the stage of exponential growth*

Sample	Date of picking	Wet wt. (g.)	Dry wt. (g.)	Dry matter (%)	Asperuloside (% of dry wt.)
1*a*	14. iv. 47	10·5	1·33	—	3·25
b		12·4	1·57	—	3·35
c		12·4	1·58	12·7	—
2*a*	18. iv. 47	12·3	1·54	—	3·25
b		12·5	1·57	—	3·35
c		12·1	1·51	12·5	—
3*a*	22. iv. 47	14·8	1·67	—	3·20
b		16·4	1·84	11·3	—
4*a*	29. iv. 47	27·8	3·6	—	3·20
b		29·2	3·8	—	3·30
c		31·1	4·0	12·9	—

Table 6. *Asperuloside in the fruit of* Galium aparine

Condition of fruit	Wet wt. of 30 fruit	Dry matter (%)	Asperuloside per fruit (mg.)	Asperuloside (% of dry wt.)
Small and soft	0·766	31	0·25	3·2
Beginning to harden	0·992	33	0·39	3·7
Hardened	0·977	37	0·44	3·6

Table 7. *Asperuloside in the leaves of* Rubia tinctorum

Whorl no.	No. of leaves	Length of internode (cm.)	Dry matter per leaf (g.)	Asperuloside (% of dry wt.)
1	6	2·6	0·003	10·4
2	6	7·0	0·015	10·8
3	6	8·0	0·054	8·4
4	7	7·0	0·055	5·0
5	6	7·5	0·045	3·7
6	6	6·5	0·047	2·4

Table 8. *Asperuloside in the phloem of* Rubia tinctorum

Strip	Wet wt. (mg.)	Dry wt. (mg.)	Dry matter (%)	Asperuloside (% of dry wt.)
1	12·0	2·35	—	23
2	10·0	1·95	—	24
3	13·3	2·60	19·5	—
4	10·2	2·0	19·5	—

Rubia tinctorum

Determinations of asperuloside in the growing shoots of the dyer's madder, *R. tinctorum*, showed that the same process of accumulation and subsequent utilization of asperuloside occurs in this plant. In this case the initial concentration of glycoside in the young leaves was much higher (10–15 % of the dry weight) than in *Galium aparine*. Figures for successive whorls of leaves, numbered from the apex of the shoot downwards, are tabulated in Table 7. The length of the accompanying internode is given as an additional index of the stage of growth.

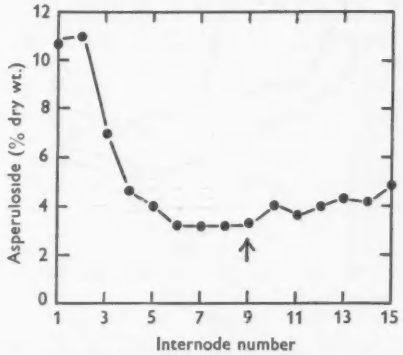


Fig. 4. The concentration of asperuloside in successive internodes of a shoot of *R. tinctorum*. Node 1 is the most apical.

The values for the asperuloside concentration in successive internodes of a developing shoot, numbered from the apex downwards, are recorded in Fig. 4. The arrow in this figure marks the ninth internode below which the asperuloside concentration rises. Comparison of these internodes with higher ones is scarcely permissible, since they had begun to develop characteristics of the subterranean parts of the plant. This was indicated by the growth of adventitious rootlets from some of the nodes and the appearance of anthraquinone glycosides, which are characteristic of the roots and underground stems of *Rubia*. The observations in the following section relate to the form of the curve in Fig. 4.

Asperuloside in the vascular tissues of the stem. A fully developed internode of *R. tinctorum* was divided into three parts: cortex, inner bark or phloem and xylem combined with pith. Tests showed that there is scarcely any asperuloside in the cortex and woody regions, but there is a very high concentration in the phloem. Fresh strips of the latter were weighed rapidly on a torsion balance, dropped into a known volume of reagent *A* and the asperuloside estimated. Other strips were taken for dry-weight estimations. More than 20 % of the dry matter was found to be asperuloside. The values are recorded in Table 8. If younger, still growing internodes are tested much asperuloside is found in the cortex and pith.

Asperula odorata

The pattern of distribution of asperuloside in *A. odorata* was found to be very similar to that in the two plants already described. An experiment with this plant was devised to confirm the observations already made. It was arranged to cover the period of flowering to see if the development of the inflorescence is accompanied by asperuloside accumulation. The experiment was started when it was just possible to separate the developing flower buds from the shoot and continued until the flowers were just beginning to die. Sixty shoots were picked at random from one large clone at intervals of 3 days over a period of 4 weeks. These batches were divided into two equal parts and the nodes of each part

separated. The leaves were separated from the stems and the inflorescence was counted as one node, number 1. The parts from one batch of 30 shoots were used for dry-weight determinations and those from the second for asperuloside determinations. In this way it was possible to follow the course of asperuloside accumulation and disappearance in each unit. The results recorded in Figs. 5*a* and *b* cells appears to be as follows: after the primary meristematic stage, the cells enter the period of exponential growth, during which the glycoside accumulates in amounts directly proportional to the increase in dry matter. This may be the result of simple storage of the product but may equally be due to the accumulation of glycoside, resulting from the balance between opposed processes of synthesis

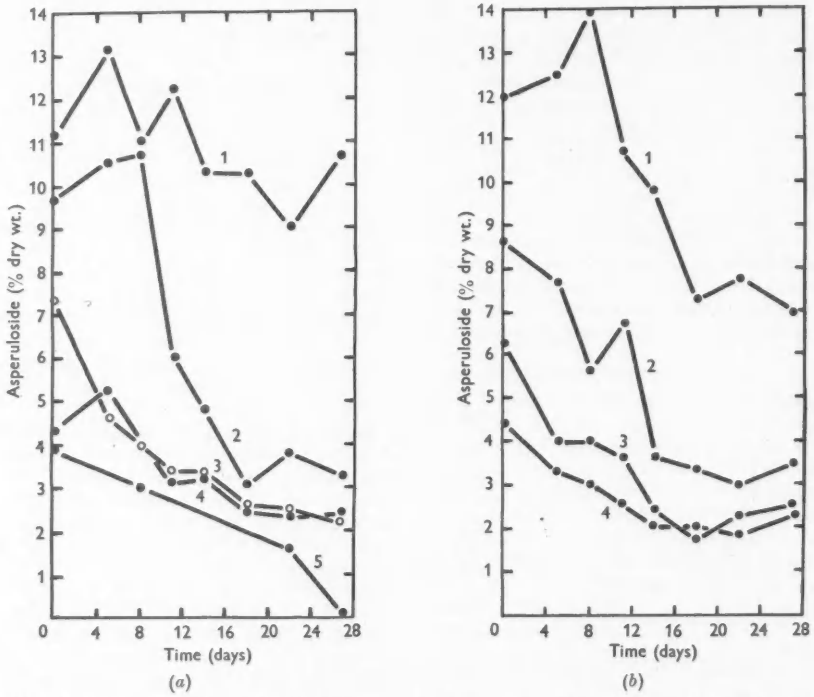


Fig. 5 (*a*, *b*). The concentration of asperuloside in components of successive nodes of shoots of *Asperula odorata* during flowering. The nodes are numbered from the apex downwards: (*a*) leaves, number 1 is the inflorescence; (*b*) internodes, number 1 corresponds to leaves number 2.

show that the parts of *A. odorata* accumulate and utilize asperuloside according to the same developmental pattern as the two plants already investigated. In the inflorescence the glycoside level remains high throughout the experiment. However, this was the only part which continued to form new elements, thus compensating for the loss of glycoside in the senescent elements.

DISCUSSION

While the study of individual cells has not been possible in this work, it appears that many of them accumulate asperuloside during an early stage of their existence. In this respect the history of such and removal, in which there is a preponderance of synthesis over removal. The disappearance of the glycoside could occur by further metabolism at or near to the site of formation, by translocation, or by a combination of both processes. At the end of exponential growth of the cells increase in volume virtually ceases and a decline in glycoside accumulation sets in, so that finally the cells become devoid of it. This change would be explained either by a cessation of synthesis or by a decline of synthesis sufficient to be completely overbalanced by a continued steady removal of glycoside, or by an increased rate of removal. Whatever the explanation, the cessation of growth is accompanied by an abrupt change in glycoside metabolism.

The chlorenchyma may not accumulate asperuloside and the epidermal cells also appear to be exceptional in that very few, if any of them, accumulate the glycoside. Since the methods employed were crude these conclusions are only tentative. In all organs part of the vascular system appears to be exceptional in continuing to accumulate asperuloside until the organ becomes moribund. The technique available did not permit a distinction between individual cells, but it is certain that this phenomenon is confined to the inner bark, that is, to the phloem and cambium. But this is a region of continued growth, containing young cells which would be expected to contain the maximum amount of asperuloside. Consequently, it might be suggested that the exceptional behaviour of these parts, in respect of asperuloside accumulation, is more apparent than real. However, it should be added that the amounts of asperuloside found in the inner bark were in excess of what might have been expected if it were composed entirely of very young cells, which is not the case. In addition, it has to be remembered that the phloem is the main path of transport for organic material, so that the large amounts of asperuloside found there may be in process of transport.

As far as the author is aware this is the fullest investigation of its kind. But there are many other recorded observations which support the view that the distribution of asperuloside in the rubiaceous plant is typical of the distribution of glycosides and many other metabolites throughout the whole range of higher plants. It is frequently stated in the literature that young, actively growing parts of a particular plant are the most abundant source of some of its characteristic products. The young shoots of many plants are pigmented by anthocyanins in the developing epidermal cells, and the pigmentation often disappears after these cells have become fully grown. In his observations on the living rose leaf, Guilliermond (1913) showed that anthocyanins accumulate in the developing vacuoles of the young epidermal cells, and thus provide a visible parallel to the first stage of the process which has been postulated to explain the present observations on asperuloside. Hurry (1930) showed that the largest amount of the indigo-producing glycoside of *Isatis tinctoria* is present in the very young leaves and that the older leaves contain much less. The classical work of Treub (1896, 1904, 1907) on cyanogenesis in *Pangium edule* and other plants provides further examples of the same developmental process.

Certain exceptional cases have been observed. Bourquelot & Danjou (1905), and others, have shown that sambunigrin, D-mandelonitrile glucoside, which occurs in the elder, *Sambucus nigra*, remains in the leaves even after they have fallen. Treub (1904) noticed the same phenomenon with the cyanogenetic glycoside of *Indigofera galegoides*. There is a suggestion in such cases that the appropriate hydrolysing enzyme is absent, but it has yet to be shown that such phenomena are not also dependent upon the absence of all of a number of different modes of utilization of the glycoside concerned. At the other extreme, Treub (1904) found that in *Phaseolus lunatus* there is a marked diurnal variation in the amount of cyanogenetic glycoside stored in the leaves.

The balance of evidence clearly favours the view that glycosides are rapidly synthesized and broken down in the plant and that in some cases at least large amounts of glycoside are involved. The characteristic distribution of these substances in the plant is related chiefly to different metabolic stages in its development. If, as it seems, glycosides enter into the metabolic processes of plants, they cannot be regarded as mere end products of metabolism, and it is important to determine the reactions in which they participate.

SUMMARY

1. The accumulation and utilization of asperuloside in the tissues of three rubiaceous plants have been studied.

2. The glycoside accumulates in young tissues such as new leaves in the stage of exponential growth and cells in the neighbourhood of the cambium.

3. The accumulation stops abruptly with the completion of the stage of exponential growth and is followed by a period of glycoside depletion, which may involve translocation to another part of the plant.

4. Nearly all the cells in the plant, with the possible exception of most of those in the epidermis and chlorenchyma of the aerial parts and some of those in the roots, appear to accumulate asperuloside when in the early stage of growth.

5. The significance of these results in relation to metabolism and translocation is discussed.

6. The general pattern of production and utilization of asperuloside appears to be characteristic of many glycosides and other metabolic products throughout the higher plants.

The author wishes to thank Dr R. Hill, for his close interest and advice, also Dr A. C. Chibnall, F.R.S., and Dr C. S. Hanes, F.R.S., for their interest in this work, which was carried out as part of the programme of the Agricultural Research Council Unit of Plant Biochemistry, Cambridge.

REFERENCES

Bourquelot, E. & Danjou, E. (1905). *C.R. Acad. Sci., Paris*, **141**, 59.
Guilliermond, A. (1913). *C.R. Acad. Sci., Paris*, **157**, 1000.
Hurry, J. B. (1930). *The Woad Plant and its Dye*, p. 43. Oxford: University Press.
Treub, M. (1896). *Ann. Jard. bot. Buitenz.* **13**, 1.
Treub, M. (1904). *Ann. Jard. bot. Buitenz.* **19**, 86.
Treub, M. (1907). *Ann. Jard. bot. Buitenz.* **21**, 79.
Trim, A. R. & Hill, R. (1952). *Biochem. J.* **50**, 310.

A Modified Medium for *Lactobacillus casei* for the Assay of B Vitamins

By K. M. CLEGG, E. KODICEK and S. P. MISTRY
Dunn Nutritional Laboratory, University of Cambridge and Medical Research Council

(*Received* 9 *March* 1951)

Snell & Wright (1941) were the first to point out that their method for the assay of nicotinic acid could be applied to the determination of biotin and pantothenic acid. Shortly afterwards, Landy & Dicken (1942) published a general procedure for the estimation of six vitamins, using *Lactobacillus casei* as the test organism. Roberts & Snell (1946) devised a medium for microbiological assays with *L. casei*, which was an improvement on the original medium of Landy & Dicken (1942).

On investigating the various factors affecting the growth of *L. casei*, we have modified the medium of Teply & Elvehjem (1945) with the aim of developing a suitable medium which could be used for assays of various vitamins.

MATERIALS AND METHODS

Stock solutions

Unless otherwise mentioned, the stock solutions were prepared according to techniques described by Snell (1950).

Salt solution E (Kodicek & Pepper, 1948*a*).

$MgSO_4.7H_2O$, 60 g.; $FeSO_4.7H_2O$, 4 mg.; $MnSO_4.4H_2O$, 1·5 g.; $CuSO_4.5H_2O$, 4 mg.; NaCl, 0·5 g.; $ZnSO_4.7H_2O$, 4 mg.

were dissolved in 250 ml. of water and 3 drops of conc. HCl added.

Casein hydrolysate: H_2SO_4-hydrolysed, vitamin-free 'Labco' casein. 'Labco' casein (200 g.) with 1 l. 25% (w/w) H_2SO_4, was autoclaved for 15 hr., at 15 lb. pressure. A litre of water was added, sulphate precipitated with about 850 g. $Ba(OH)_2$, the mixture autoclaved for 10 min. at 10 lb. pressure, and filtered hot through a Büchner funnel. The $BaSO_4$ cake was thoroughly broken up in 1 l. water, autoclaved and filtered hot. The precipitate was washed once more as before. The combined filtrates were heated and the excess barium was carefully precipitated with 25% H_2SO_4. The suspension was filtered hot, and the filtrate concentrated on a water bath to about 2500 ml. Dry weight determination gave a recovery of a little over 90%.

The hydrolysate at pH 6·0 was stirred for 1 hr. with 100 g. Norite charcoal (British Drug Houses Ltd.), filtered and the pH adjusted to 3·8 with glacial acetic acid. The charcoal treatment was repeated, the solution filtered and brought to pH 6·8–7·0 with 40% KOH. The hydrolysate was finally treated with Norite, 40 g., for 1 hr. The residue from each filtration was washed with 50–100 ml. of water and the washings added to the filtrate. The filtrate was concentrated on a water bath to contain 10% (w/v) casein solids. Recovery was 73% (casein solids). Before use, new batches of hydrolysate were always tested at several levels and the optimum concentration determined. An amount of 50 mg./10 ml. medium was usually found adequate. We have found it necessary to restandardize the adsorption treatment when a different brand of charcoal was used.

Peptone treated with Norite charcoal. 'Difco' bactopeptone (10 g.) was dissolved in about 80 ml. water, pH adjusted to 3·0 with HCl and made up to 100 ml. The solution was stirred with 5 g. Norite for 1 hr., filtered, and the charcoal treatment repeated twice with 2 g. Norite.

Pteroylglutamic acid (PGA). During the early part of the work 'Folvite' (Lederle Laboratories) was used as the PGA standard. Later, when a 97% pure and aldehyde-free preparation (Lederle Laboratories) became available it was used instead.

A stock solution of PGA containing 100 μg./ml. was prepared in 1% K_2HPO_4. It was renewed twice weekly. From the stock solution weaker standards were prepared for immediate use.

Bacteriological procedure

The double-strength medium, 5 ml., the composition of which is shown in Table 1, was diluted to 10 ml. with distilled water after adding the test solutions. The test substances were assayed usually in triplicate at each level. The tubes were placed in Petri dish canisters and autoclaved for 6 min. at 15 lb. pressure. Autoclaving for a longer period resulted in caramelization and consequent deterioration of the medium. On autoclaving, a slight precipitate resulted, but this redissolved on shaking at room temperature.

Stock culture. Cultures of *L. casei*, A.T.C.C. no. 7469, with characteristics reported by Kodicek & Pepper (1948*b*) were maintained on slopes of 'Difco' yeast-dextrose-agar supplemented with 'Hepamino' liver powder (Evans) 1% and glucose 0·5%. They were subcultured every 2–3 weeks. Each subculture consisted of two transfers from liquid to

liquid medium and incubation at 37° for 16–20 hr. The medium (see Table 1) supplemented with 1% 'Hepamino' liver powder was used. After the second transfer, slopes were prepared by loop transfers from the centrifuged bacterial sediment, incubated for 20–24 hr. at 37° and stored in the refrigerator at 4°.

Table 1. *Basal medium for* L. casei*

(The amounts refer to a concentration in 10 ml. of single strength medium. Normally a double strength medium was prepared and finally diluted with water or test solution. Norite-treated peptone is omitted for biotin and tryptophan assays. The pH was adjusted to 6·8.)

Casein (Labco), H_2SO_4-hydrolysed, hydrolysate	50·0 mg.
Peptone, Norite-treated	2·0 mg.
Glucose	300·0 mg.
Potassium acetate	300·0 mg.
DL-Alanine	1·0 mg.
L-Cystine	2·0 mg.
DL-Tryptophan	2·0 mg.
Adenine, guanine, uracil and xanthine (each)	0·1 mg.
Aneurin chloride hydrochloride	10·0 μg.
Riboflavin	10·0 μg.
Nicotinic acid	10·0 μg.
Pyridoxin hydrochloride	25·0 μg.
Calcium-D-pantothenate	10·0 μg.
Biotin (free acid)	0·04 μg.
p-Aminobenzoic acid	2·0 μg.
Pteroylglutamic acid	0·1 μg.
K_2HPO_4 and KH_2PO_4 (each)	25·0 mg.
Salt solution E	0·05 ml.

* The composition of the medium has been communicated to the Biochemical Society (Kodicek & Mistry, 1949*a*).

Preparation of the inoculum. For the inoculum, a procedure similar to that adopted for the subculturing was used, except that after the second transfer from liquid to liquid medium the centrifuged bacterial sediment was thoroughly washed two or three times and resuspended in saline.

The inoculum was standardized to contain 30 μg. dry weight bacteria per ml. and 2 drops were seeded aseptically into each assay tube. This inoculum contained about 2×10^6 viable cells as determined by a plate count.

Estimation of growth. The assay tubes were incubated in their racks at 37° for 72 hr. and growth was estimated by determining acid produced. The cultures were titrated with 0·1 N-NaOH, using bromothymol blue as internal indicator. The tubes were not disturbed during the incubation period.

RESULTS

Effect of peptone. Peptone appreciably improved growth in the presence of PGA. With all the levels tested the acid production in the blanks remained the same, indicating that the preparation was practically free from PGA. A concentration of 2 mg. was adopted for the medium.

The effect of peptone was finally investigated at several concentrations of PGA and the results are shown in Table 2. In the presence of peptone a better agreement between replicates was observed.

The other constituents, namely, salts, glucose, cystine, tryptophan, alanine, purine and pyrimidine solution and B vitamins, were tested individually in increasing amounts and a concentration allowing for optimal growth was adopted for the medium (Table 1). Asparagine was not included in the medium since it retarded growth (Kodicek & Mistry, 1949*b*).

Table 2. *Effect of Norite-treated peptone on PGA dose response*

PGA (mμg./10 ml.)	Final medium: No peptone	Final medium: With 2 mg. peptone/10 ml
	0·1 N-acid produced (ml./10 ml.)	
0·0	3·4	3·2
0·1	4·2	5·6
0·2	5·2	8·8
0·4	7·1	12·8
0·6	8·6	13·7
0·8	9·8	16·2
1·0	10·6	17·7
2·0	16·3	20·2
100·0	23·1	28·2
Liver control*	30·4	30·8

* Hepamino 100 mg./10 ml.

The assay of pteroylglutamic acid

The titrimetric dose response curve is shown in Fig. 1. When compared with the published results of Teply & Elvehjem (1945) and Roberts & Snell (1946), the medium gave a better response per unit of the vitamin added. The turbidimetric response curve after 42 hr. incubation is shown in Fig. 2. Shorter incubation periods were not satisfactory. However, the titrimetric method was preferred as it was simple and convenient.

The reproducibility of the results are shown in Table 3. The standard deviation, calculated for different levels of PGA, varied from ±0·431 to ±0·856, and the coefficient of variation ranged from about 3 to 13%.

Tests with PGA conjugates and related substances. Pteroic acid, rhizopterin, Diopterin (Pteroyl-α-glutamylglutamic acid), Teropterin (pteroyl-γ-glutamyl-γ-glutamylglutamic acid), and vitamin B_c conjugate (pteroylhexaglutamylglutamic acid) were tested at several levels.

Pteroic acid and rhizopterin were essentially inactive for *L. casei* as was reported previously (Jukes & Stockstad, 1948). Compared with PGA, Diopterin and Teropterin had on a molar basis an activity of 0·5 and 38%, respectively. The activity of the former agreed with that reported by Semb, Boothe, Angier, Waller, Mowat, Hutchings & Subbarow (1949), but Teropterin was found to be about 60% less active. On the other hand, B_c conjugate was shown to have 4·6% activity compared to 0·9–1·8% reported by Waller *et al.* (1948).

4-Hydroxypteridine, 2-amino-4-hydroxypteridine, 2-amino-4-hydroxy-6-pteridine carboxylic acid and 2-amino-4-hydroxy-7-pteridine carboxylic acid were tested individually at a concentration of 100 μg./10 ml. medium in absence and presence of PGA. None of them showed any activity, nor did they have any stimulatory or inhibitory effect in the presence of PGA.

The assay of other B vitamins and tryptophan

The medium was used for the assay of other B vitamins and the results are given below. The vitamin or amino-acid to be determined was omitted from the medium and was tested in graded amounts. Biotin and tryptophan were assayed in absence of

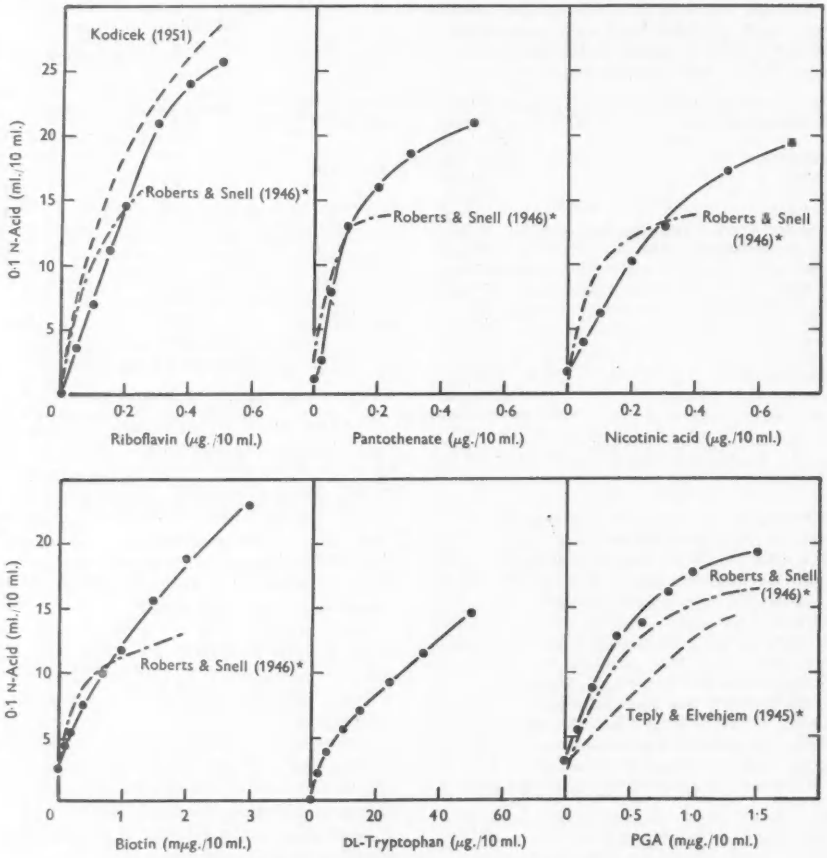


Fig. 1. Microbiological assays with *L. casei* (72 hr. titrimetric assay).
* Reproduced from original publications.

Table 3. *Response of* L. casei *to increasing concentrations of PGA*

Concn. of PGA (mμg./10 ml. medium)	No. of tubes	0·1 N-acid produced (ml.) Mean	Standard deviation	Coefficient of variation (%)
0	11	3·2	±0·431	13·4
0·2	11	8·8	±0·856	9·7
0·6	11	13·7	±0·702	5·1
1·0	11	17·7	±0·451	2·6

Norite-treated peptone. The results are shown in Fig. 1. For comparison, the dose-response curves from the published data of Roberts & Snell (1946) and Kodicek (1951) are also shown.

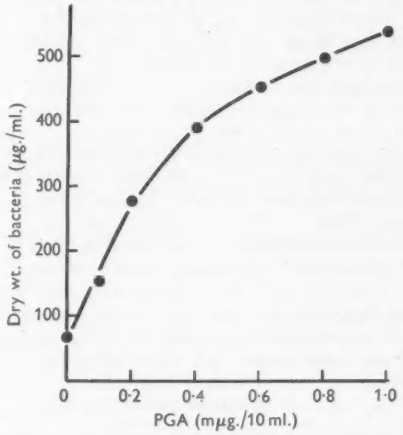


Fig. 2. PGA dose response curve with *L. casei* (42 hr. turbidimetric assay).

Nicotinic acid

As will be seen from Fig. 1, small amounts of nicotinic acid gave lower responses than those obtained with Roberts & Snell's (1946) medium. However, with the present medium the vitamin could be assayed over a much wider range of concentrations and the acid production using *L. casei* was higher than that reported by Kodicek & Pepper (1948*a*) with *L. arabinosus*. The medium has been repeatedly tested and the dose response curves showed little variation from experiment to experiment. The agreement between replicates at various levels of nicotinic acid was satisfactory (Table 4).

Extraction of nicotinic acid from test materials. A suitable extraction procedure for most cereals was found to be hydrolysis with N-NaOH for 45 min. on a water bath, and for other foodstuffs, treatment with N-H_2SO_4 at 15 lb. pressure for 1 hr., as described by Kodicek & Pepper (1948*b*). All extracts were washed with $CHCl_3$. This technique ensured a satisfactory extraction of active materials, and liberation of free nicotinic acid which was especially important with cereals which are known to contain a bound form of nicotinic acid (Krehl & Strong, 1944). For the assay of bound nicotinic acid, the material was extracted with 0·1 N-HCl for 1 hr. at 100°. This procedure did not liberate nicotinic acid from its bound form.

Table 4. *Variations between tubes in the assay of nicotinic acid*

Concn. of nicotinic acid (μg./10 ml. medium)	No. of tubes	0·1 N-acid produced (ml.)	Standard deviation	Coefficient of variation (%)
0	11	1·36	±0·13	9·6
0·10	14	4·88	±0·25	5·2
0·15	16	7·31	±0·27	3·7
0·20	16	9·06	±0·16	1·8
0·30	16	11·64	±0·22	1·9
0·50	16	15·50	±0·32	2·1

Table 5. *Comparison of microbiological and chemical estimations of nicotinic acid*

	Nicotinic acid					
	Microbiological method			Chemical method (Wang & Kodicek, 1943)		
Material	No. of estimations	Average result (μg./g.)	Standard error	No. of estimations	Average result (μg./g.)	Standard error
Yeast	8	221·0	±5·52	10	224·3	±13·74
Groundnuts	4	166·0	±3·89	6	167·7	±2·68
Kipper (dehydrated)	4	117·0	±3·19	6	122·0	±2·00
Muscle (beef, dehydrated)	8	103·0	±3·46	15	89·6	±4·83
Herring (dehydrated)	4	84·9	±1·79	6	81·1	±1·32
Malt extract, liquid	4	66·6	±1·85	6	64·3	±1·09
Whole wheat (ground)	4	46·8	±0·68	6	49·4	±0·50
Cocoa	4	18·2	±0·11	6	17·7	±0·65
Wheat meal flour (85% extraction)	7	17·0	±0·70	9	15·2	±0·56
Maize meal	4	13·1	±0·23	6	13·3	±0·20
Milk (dehydrated)	9	6·2	±0·14	8	4·8	±0·34
Tomatoes (fresh)	3	4·2	±0·07	5	3·6	±0·13

The results were calculated by using an 'internal standard' technique as described by Kodicek & Pepper (1948*b*).

The method has been applied to the estimation of nicotinic acid in a variety of foodstuffs and the results are shown in Table 5. It will be seen that the microbiological results are in good agreement with those obtained by a chemical procedure (Wang & Kodicek, 1943).

A number of brans has been assayed by this procedure to ascertain if *L. casei* responds to the bound form of nicotinic acid present in cereals (Table 6). It was found that the maximum response was obtained on alkaline hydrolysis, while 0·1 N-hydrochloric acid extracts of wheat, rice and barley brans showed 10–20 % activity.

Table 6. *Microbiological assay of nicotinic acid in hydrolysed and unhydrolysed samples of various brans*

	Nicotinic acid (μg./g.)		
	Microbiological method		Chemical method (Wang & Kodicek, 1943)
Bran	Unhydrolysed	NaOH-hydrolysed	NaOH-hydrolysed
Rice	54·5	255	251
Wheat	42·9	219	204
Barley	36·9	202	233
Maize	19·1	31·8	29·6
Rye	9·8	19·1	20·4

On the other hand, when 0·1 N-hydrochloric acid extracts of maize and rye brans were tested, more than 50 % of the total nicotinic acid activity was found. This may indicate either, that the bound form in maize and rye is different from the other brans tested, or that there was some free nicotinic acid in the extracts. The low activity for *L. casei* of the bound form of nicotinic acid was confirmed by testing a concentrate prepared according to Chaudhuri & Kodicek (1950) from wheat bran which contained by chemical estimation 35 mg. free nicotinic acid per gram of material. The microbiological results for the unhydrolysed and the hydrolysed material were 2·8 and 35·2 mg./g. respectively.

Riboflavin

The standard curve obtained with the present medium (Fig. 1), though quite satisfactory, was low compared to those of Roberts & Snell (1946) and Kodicek (1951). However, Kodicek's (1951) method was specially developed for the assay of riboflavin, and uses a semi-synthetic medium containing treated peptone and yeast extract which supplied additional growth stimulatory factors.

Calcium-D-pantothenate

The standard curve (Fig. 1) compared favourably with that of Roberts & Snell (1946). Except for a few assays on extracts of natural materials the medium has not been used for routine estimations of the vitamin.

Biotin and biocytin

The dose-response curve for biotin was considerably better (Fig. 1) than that of Roberts & Snell (1946). The linear portion of the curve extended up to 2 mμg. biotin/10 ml. medium.

Investigations of earlier workers indicated the existence of a complex of biotin in natural materials. Recently a bound form of the vitamin, termed biocytin, has been isolated in a crystalline form from yeast extract and its biological characteristics have also been described (Wright, Cresson, Valentik & Skeggs, 1950).

A concentrate of biocytin was tested on the basis of its biotin content in increasing concentrations from 0 to 5 mμg./10 ml. For comparison, crystalline biotin (free acid) was also tested at the same levels. At all concentrations crystalline biotin and the biocytin concentrate had essentially the same activity.

Tryptophan

A satisfactory linear response was obtained (Fig. 1) over a wide range of concentrations. Since only *L. arabinosus* has been used for the assay of this amino-acid by other workers, no comparative data can be given.

DISCUSSION

The medium, though primarily developed for the determination of pteroylglutamic acid with *L. casei*, was also found satisfactory for other B vitamins.

Most published media for the assay of nicotinic acid, pantothenic acid, and biotin, have preferred *L. arabinosus* to *L. casei* as the test organism (Snell, 1950). The choice of *L. arabinosus* was influenced by its simpler nutritional requirements. However, of the two organisms, *L. casei* has a greater acid-producing potential which increases the convenience and accuracy of titrations, and is also more sensitive to small increases in the vitamin under test, provided that the medium employed is optimal or at least near optimal. The proposed procedure is convenient in that a single basal medium and a single organism suffice for an assay of five vitamins and tryptophan. Furthermore, the same medium with minor modifications has been used for the assay of strepogenin activity (Mistry & Kodicek, 1951).

The medium showed good possibilities for the assay of biotin. Biocytin, a naturally occurring bound form of biotin, was active for *L. casei* which confirmed the findings of Wright, Cresson, Skeggs, Wood, Peck, Wolf & Folkers (1950).

Nicotinic acid has been almost exclusively assayed with *L. arabinosus* as the test organism. Of the more

recent methods, that described by Kodicek & Pepper (1948*b*) had been in use in this laboratory, but the present medium with *L. casei* has completely replaced the former procedure and was found to give more satisfactory results.

In the assay of pteroylglutamic acid the conjugates, with the exception of Teropterin, showed an activity in general agreement with the findings of other workers.

SUMMARY

1. An improved medium was developed for the assay of pteroylglutamic acid with *Lactobacillus casei* and was tested for some other B vitamins and tryptophan.

2. The titrimetric dose-response curves for riboflavin, nicotinic acid, calcium pantothenate, biotin and tryptophan were satisfactory.

3. The medium has been used in particular for the microbiological assay of total nicotinic acid, and the results were in good agreement with the chemical values. The bound form of nicotinic acid present in cereals was found to be less active than the free vitamin.

4. A number of conjugates of pteroylglutamic acid and other pteridines were tested.

We wish to thank Dr A. Albert for the synthetic pteridines, Dr T. H. Jukes for pteroylgutamic acid and conjugates, Dr J. J. Pfiffner for the vitamin B_c conjugate, Dr L. D. Wright for the biocytin concentrate, Dr K. Folkers for biotin and rhizopterin, and the Flour Millers Association for the supply of the different brans.

REFERENCES

Chaudhuri, D. K. & Kodicek, E. (1950). *Nature, Lond.*, **165**, 1022.
Jukes, J. H. & Stokstad, E. L. R. (1948). *Physiol. Rev.* **28**, 51.
Kodicek, E. (1951). In preparation.
Kodicek, E. & Mistry, S. P. (1949*a*). *Biochem. J.* **45**, xxxv.
Kodicek, E. & Mistry, S. P. (1949*b*). *1st Int. Congr. Biochem. Abstr.*, p. 343.
Kodicek, E. & Pepper, C. R. (1948*a*). *J. gen. Microbiol.* **2**, 292.
Kodicek, E. & Pepper, C. R. (1948*b*). *J. gen. Microbiol.* **2**, 306.
Krehl, W. A. & Strong, F. M. (1944). *J. biol. Chem.* **156**, 1.
Landy, M. & Dicken, D. M. (1942). *J. Lab. clin. Med.* **27**, 1086.
Mistry, S. P. & Kodicek, E. (1951). *Biochem. J.* **49**, xviii.
Roberts, E. C. & Snell, E. E. (1946). *J. biol. Chem.* **163**, 499.
Semb, J., Boothe, J. H., Angier, R. B., Waller, C. W., Mowat, J. H., Hutchings, B. L. & Subbarow, Y. (1949). *J. Amer. chem. Soc.* **71**, 2310.
Snell, E. E. (1950). *Vitamin Methods*, Vol. I, p. 327. New York: Academic Press Inc.
Snell, E. E. & Wright, L. D. (1941). *J. biol. Chem.* **139**, 675.
Teply, L. J. & Elvehjem, C. A. (1945). *J. biol. Chem.* **157**, 303.
Waller, C. W., Hutchings, B. L., Mowat, J. H., Stokstad, E. L. R., Boothe, J. H., Angier, R. B., Semb, J., Subbarow, Y., Cosulich, P. B., Fahrenbach, M. J., Hultquist, M. E., Kuh, E., Northey, E. H., Seeger, D. R., Sickels, J. P. & Smith, J. M. jun. (1948). *J. Amer. chem. Soc.* **70**, 19.
Wang, Y. L. & Kodicek, E. (1943). *Biochem. J.* **37**, 530.
Wright, L. D., Cresson, E. L., Skeggs, H. R., Wood, T. R., Peck, R. L., Wolf, D. E. & Folkers, K. (1950). *J. Amer. chem. Soc.* **72**, 1048.
Wright, L. D., Cresson, E. L., Valentik, K. & Skeggs, H. R. (1950). *Fed. Proc.* **9**, 374.

Specificity of Glucose Oxidase (Notatin)

By D. KEILIN and E. F. HARTREE
Molteno Institute, University of Cambridge

(*Received* 23 *May* 1951)

The antibiotic 'notatin' isolated in a very highly purified state from culture medium filtrates of *Penicillium notatum* by Coulthard, Michaelis, Short, Sykes, Skrimshire, Standfast, Birkinshaw & Raistrick (1942, 1945) was shown by these authors to be identical with the glucose oxidase discovered by Müller (1928, 1941) in cultures of different moulds (for a summary of the literature, see Keilin & Hartree, 1948*a*). The enzyme is a flavoprotein (Coulthard *et al.* 1945) and the prosthetic group has been identified as alloxazine-adenine-dinucleotide (Keilin & Hartree, 1946).

A study of the catalytic activities of this enzyme revealed its very high specificity for glucose (Keilin & Hartree, 1948*a*) which is quantitatively oxidized to gluconic acid. Previous workers had shown that the reaction proceeds according to the equation

$$C_6H_{12}O_6 + O_2 + H_2O = C_6H_{12}O_7 + H_2O_2.$$

In presence of catalase, which decomposes the peroxide to oxygen and water, the overall reaction is

$$C_6H_{12}O_6 + \tfrac{1}{2}O_2 = C_6H_{12}O_7.$$

Further experiments showed that notatin could be successfully used for manometric estimation of glucose in biological material in presence of other carbohydrates and proteins, and that the method could be extended to studies of the kinetics of different enzyme reactions which yield glucose (Keilin & Hartree, 1948*b*).

Of about fifty other sugars and their derivatives tested only mannose, xylose, 6-methyl glucose and 4:6-dimethyl glucose were oxidized, although the rates of oxidation were only 1–2% that of glucose, whereas the remainder were practically unoxidized. This investigation has now been extended to the complete range of aldohexoses with the result that notatin can be considered as strictly specific for glucose.

Early in 1949 it was suggested to us by Dr A. Gottschalk that it would be interesting to determine whether the specificity of notatin extends to the α- and β-forms of glucose. It would seem reasonable to assume that one form would be more actively oxidized since stereospecificity is a general property of enzymes. Such a specificity for the α-configuration has been shown by Cori & Cori (1940) and by Campbell & Creasy (1949) who found that the α-form of glucose is the specific inhibitor of the synthesis by phosphorylases of polysaccharides from glucose-1-phosphate which itself has the α-configuration. Even chemical oxidizing agents react at different rates with the two isomers. Thus bromine (Isbell & Pigman, 1933) and hypoiodous acid (Reeve, 1951) will, under certain conditions, oxidize β-glucose 35 and 25 times respectively more rapidly than the α-isomer.

Experiments with notatin have shown that β-glucose is the more rapidly oxidized isomer and that the rate of oxidation of α-glucose is very slow indeed when the necessary corrections for mutarotation have been applied. While our experiments were in progress we learned from Dr A. Neuberger that an independent study, the results of which have since been published (Bentley & Neuberger, 1949), also indicated that β-glucose was the more readily oxidized form.

EXPERIMENTAL

Materials

Notatin. Samples of notatin used in this investigation were supplied by the Boots Pure Drug Company through the courtesy of Dr W. F. Short. They were samples of two different batches labelled '410–417' and '233–237' which we shall denote as *Y* and *Z* respectively in this paper. The amount of water-insoluble material in these samples was *Y*, 37% and *Z*, 8%. However, the glucose oxidase activities of the two samples were equal in terms of the soluble fraction (Q_{O_2}, 40,000 μl./mg. dry wt./hr. in air at 20° and pH 5·6). The weights of notatin given in the text refer to the dry weight of the soluble material.

Catalase was obtained from horse-liver by a method previously described (Keilin & Hartree, 1945). All traces of ethanol had been removed by prolonged dialysis. The solution was 0·44 mM with respect to haematin.

Sugars. α- and β-Glucoses were prepared according to Hudson & Dale (1917). These sugars were normally dried in vacuum over P_2O_5 immediately after their preparation and so obtained anhydrous. One air-dried sample of α-glucose was found on analysis to be the monohydrate. (Found: C, 36·5; H, 7·34. Calc. for $C_6H_{12}O_6.H_2O$: C, 36·3; H, 7·16%. 302 mg. lost 26·3 mg. on heating at 105° for 24 hr. Calc. for $1H_2O$: 27·5 mg.) Whenever the monohydrate was used the weight recorded is the equivalent of anhydrous glucose. The specific mutarotations given in Table 1 agree closely with those of Hudson & Yanovsky (1917). For the study of equilibrium glucose, a 1% aqueous solution was brought to equilibrium by heating 10 min. at 100°.

Table 1. *Specific rotations of samples of α- and β-glucose used in the present investigation*

(4% (w/v) solutions of glucose in water at 20°; 22 cm. tube.)

Glucose isomer	Initial specific rotation (°)	Equilibrium specific rotation (°)
β-Glucose	17·8	52·5
α-Glucose	111·4	52·5
α-Glucose monohydrate	100·2	47·6
α-Glucose monohydrate after heating at 105° for 24 hr.	110·0	52·3

As to other sugars used in the present study, a sample of D-altrose was supplied by Prof. C. S. Hudson, D-allose, D-talose and D-idose (syrup) were supplied by Prof. T. Reichstein and samples of D-talose and D-gulose ($CaCl_2$ derivative) were supplied by Dr H. S. Isbell. 4:6-Benzylidene glucose was prepared according to Zerfas (1931).

Phosphate buffer in all experiments was a mixture of 920 ml. 0·2 M-KH_2PO_4 and 80 ml. 0·2 M-Na_2HPO_4. Its pH, measured with glass electrode at 20°, was 5·61 which is the optimum for notatin.

Manometric methods

The oxidation of glucose by notatin was followed in Barcroft differential manometers at 20°. Except where otherwise stated, the right-hand flasks received notatin and 0·04 ml. catalase dissolved in 3·3 ml. buffer, while the glucose was added from a dangling cup after temperature equilibration. The left-hand flasks received 3·3 ml. buffer.

When using more than 100 μg. notatin in a manometer flask the rate of O_2 uptake, especially with β-glucose, is very rapid and in order to reduce experimental error all measurements, with the exception of those recorded in Fig. 4, were carried out in quadruplicate with rapid shaking of manometers (150/min.). Little advantage can be gained by working at 0° since not only mutarotation but also the oxidation catalysed by notatin drops considerably.

The α- and β-glucoses were weighed into dangling cups in the dry form, and since the most convenient quantity for a Barcroft manometer is about 3 mg. it was scarcely practical as a routine measure to weigh this out to an accuracy (1–2%) which would be within the experimental

error. The sugars were therefore weighed out to within 10% of 3 mg., and the periodical readings of a set of four identically filled manometers were converted to percentage of final readings (corresponding to complete oxidation) before being averaged. In presence of catalase the oxidation of 3 mg. glucose requires 187 μl. oxygen. The results are expressed as plots of $\log_{10}$% glucose unoxidized against time (see Kinetics).

Polarimetric methods

Mutarotation was studied in a 22 cm. jacketed tube in which the temperature was maintained at 20±0·05°. For measurements of rotation in absence of notatin the glucose was weighed into a 15 ml. volumetric flask and made up with water or buffer to the mark at zero time. Immediately after transfer of the solution to the polarimeter tube the readings of rotation were begun and a series of about a dozen was made within 30–40 min. (excluding the final reading) when buffer was used as solvent; with water the experiment was extended for a longer period. A sodium lamp was used as light source.

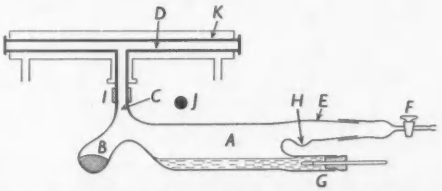


Fig. 1. Arrangement for filling a 22 cm. jacketed polarimeter tube with a solution of glucose and notatin in buffer under anaerobic conditions. For explanation of letters see text.

For the study of mutarotation in presence of notatin the experiments had to be carried out under strictly anaerobic conditions in order to avoid oxidation of the glucose. This was achieved by means of the apparatus shown in Fig. 1. The tube *A* of circular section was drawn out at one end to a bulb *B* and to a side arm *C* of the same diameter as the filling orifice of the polarimeter tube *D*. To the other end of *A* were fused two parallel narrower tubes: *E* which carried a standard ground joint and tap *F*, and *G* which held a thermometer in a rubber stopper. The small depression *H* was used in experiments to be described in a later paper (Keilin & Hartree, 1952).

By having *A* clamped vertically it was possible to introduce a known weight of glucose via *C* into *B*. The polarimeter tube, the end plates of which had been sealed on with 'picein' wax, was then attached by means of a rubber sleeve *I* and the entire apparatus clamped horizontally as shown in Fig. 1 to a retort-stand clamp which is shown in cross-section at *J*. The solution of notatin in buffer was introduced into *A* via the standard joint of the branch *E* the tap being then fixed in position with vacuum grease.

Air was removed from the system by threefold evacuation and filling with N_2 that had been purified over hot copper, the apparatus being gently rocked about *J* to assist the removal of dissolved O_2. The jacket *K* of the polarimeter tube *D* was connected to a constant-temperature circulating system by tubing flexible enough to allow the transfer of the liquid in *A* to the polarimeter tube by the following procedure: when the notatin solution in *A* had been brought to about 20° (the warmth of the hand sufficed) the apparatus was tilted to and fro about *J*, through 90° each way, until the glucose had dissolved (zero time). Then, by turning the apparatus through 180° so that *A* was horizontal and uppermost, and rocking it gently, the solution, free from air-bubbles, could be rapidly passed into the polarimeter tube. The clamp *J* was so placed that at this stage the polarimeter tube could be readily lowered into the polarimeter trough and a series of rotation readings begun within 1·5 min. of mixing the glucose with the notatin solution.

Notation. In order to avoid confusion which might arise in using α to denote one of the forms of glucose and also the optical rotation, the symbols α and $[\alpha]$ as normally used in polarimetry will be replaced in this paper by θ and $[\theta]$, together with suffixes α, β and eq. to denote the isomers and the equilibrium mixture.

Kinetics

Oxidation of glucose. The oxidations of α- and β-glucose catalysed by notatin under the standard manometric conditions can be expressed by the equation

$$K' = \frac{1}{t} \ln \frac{a}{a-x},$$

where K' is the velocity constant of a first order reaction (K' being used to avoid confusion with the mutarotation constant K). This equation can be rewritten

$$\log_{10} \frac{a-x}{a} = -\frac{K'}{2{\cdot}303}\, t.$$

Hence, by plotting log % sugar unoxidized against time, a straight line is obtained with slope $= -\frac{K'}{2{\cdot}303}$; the velocity constants for oxidation of isomers α and β will be denoted by K'_α and K'_β respectively.

Mutarotation of glucose. If k_α and k_β are the velocity constants in the equilibrium α-glucose $\underset{k_\beta}{\overset{k_\alpha}{\rightleftharpoons}}$ β-glucose and K is the first order mutarotation constant, it can be shown that

$$K = \frac{1}{t} \ln \frac{\theta_0 - \theta_{eq.}}{\theta_t - \theta_{eq.}} = k_\alpha + k_\beta.$$

Since no measurement at zero time is possible the more general equation

$$K = \frac{2{\cdot}303}{t_2 - t_1} \log_{10} \frac{\theta_1 - \theta_{eq.}}{\theta_2 - \theta_{eq.}}$$

has been used in which θ_1 represents the first reading and θ_2 the ten or more subsequent readings. For each experiment only the mean value of K and the standard deviation have been given. The calculation of $[\theta]_0$ for each glucose isomer, i.e. $[\theta]_\alpha$ and $[\theta]_\beta$ enables the values of k_α and k_β to be determined since $k_\alpha + k_\beta = K$ and $\frac{k_\alpha}{k_\beta} = \frac{[\theta]_\alpha - [\theta]_{eq.}}{[\theta]_{eq.} - [\theta]_\beta}$.

Effect of phosphate buffer on mutarotation of glucose

As shown in Table 2, mutarotation in presence of 0·2 M-phosphate buffer pH 5·6 is more than four times faster than in distilled water. The figures obtained for mutarotation constants in water agree

with those of Richards, Faulkner & Lowry (1927) whose average figure was 0·0146 and also with those of Isbell & Pigman (1937) when their results are converted to a natural logarithm basis. According to Hudson & Dale (1917) K is almost independent of glucose concentration provided the latter does not exceed 10%. In phosphate buffer

$$K = k_\alpha + k_\beta = 0{\cdot}0664 \text{ min.}^{-1}$$

and since we could find no significant differences between the specific rotations in buffer and in water the data of Table 1 have been substituted in the equation above to give a value of 1·70 for k_α/k_β. From these figures it can be calculated that $k_\alpha = 0{\cdot}0418$ min.$^{-1}$ and that 64% β-glucose is present at equilibrium in the phosphate buffer.

Table 2. *Mutarotation constants at 20° of α- and β-glucose in 0·2 M-phosphate buffer, pH 5·6 and in water*

(22 cm. tube. S.D. = standard deviation (%).)

Isomer	Concentration (%, w/v)	Solvent	$10^3 K$ (min.$^{-1}$)	S.D.
α	3·98	Buffer	65·8	0·84
α	2·03	Buffer	66·6	1·11
α	0·803	Buffer	66·6	5·4
β	4·01	Buffer	66·4	1·11
α	4·01	Water	14·51	0·94
β	4·00	Water	14·56	1·15

Mean $10^3 K$ in buffer: **66·4** min.$^{-1}$.

Oxidation of α- and β-glucose by notatin

The study of the oxidation of α- and β-glucose by notatin is complicated by mutarotation of both forms which is already more than four times faster in phosphate than in water and which may be further accelerated by a factor present in some samples of notatin.

The amount of notatin used in manometric experiments can be so adjusted that the enzyme is fully saturated by substrate and in this case the initial rate of O_2 uptake with either β- or equilibrium glucose will be proportional to the amount of enzyme present and from such an experiment the Q_{O_2} can be calculated. The conditions are satisfied when 10 μg. notatin is used in presence of 0·1 M-substrate.

For the comparative study of the behaviour of notatin towards α- and β-glucose, however, it becomes necessary to use much lower concentrations of glucose (~0·005 M) in order that the oxidation can be followed manometrically to completion and also to avoid a too rapid formation of the other isomer.

Oxidation of glucose catalysed by notatin Y ('410–417')

The comparative study of the oxidation of α-, β- and equilibrium glucose catalysed by notatin Y was carried out manometrically as described above (see Methods) in the presence of 80 μg. enzyme and 3 mg. of the corresponding forms of glucose.

The results of these experiments summarized in Fig. 2 clearly show that notatin catalyses the oxidation of β-glucose much more rapidly than that of α-glucose. In the case of equilibrium glucose

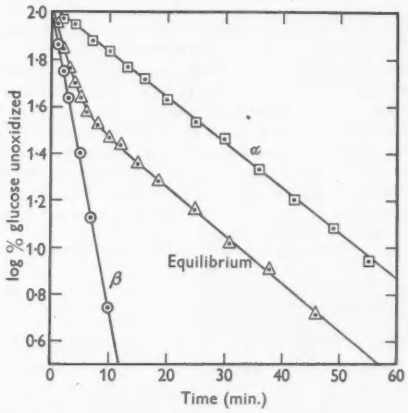


Fig. 2. The oxidation of α-, β- and equilibrium glucose by notatin Y. 80 μg. notatin, 0·04 ml. catalase, 3 mg. glucose as indicated (from dangling cup: α- and β-glucose in dry state, equilibrium glucose as 1% solution), 0·2 M-phosphate buffer (pH 5·6) to 3·3 ml. Experiments carried out in Barcroft differential manometers in quadruplicate and averaged after determination of % sugar unoxidized at each time interval. $T = 20°$. $\log \frac{(a-x)}{a} = -\frac{K'_\beta}{2{\cdot}303} t$; or log % glucose unoxidized $= 2 - \frac{K'_\beta}{2{\cdot}303} t$.

there is a break in the line corresponding to the change from β to α oxidation. This inflexion is reached within 5–7 min. during which time, since the β-form is being rapidly removed, the α to β change will predominate over the reverse reaction. The straight line obtained for α-glucose must represent the combined rates of mutarotation of α-glucose to the readily oxidizable β-glucose and any direct oxidation of α-glucose.

Oxidation of β-glucose by notatin Y. The oxidation of 3 mg. β-glucose by various amounts (10–300 μg.) of notatin Y is represented in Fig. 3. The change of slope of the logarithmic plot in experiments with 20 and 40 μg. notatin is presumably due to the fact that sufficient α-glucose is formed during the first part of the experiment to modify the rate of O_2 uptake at a later period when the mutarotation $\alpha \rightarrow \beta$ becomes the limiting factor in the reaction. With only 10 μg. notatin present, the straight line obtained indicates that the rate of oxidation of

β-glucose is not faster than that of mutarotation so that if an appreciable amount of α-glucose is formed the reverse reaction α- → β-glucose is if anything faster than the oxidation of β-glucose. In other words, with 10 μg. notatin at no time during the reaction does the mutarotation become a limiting factor.

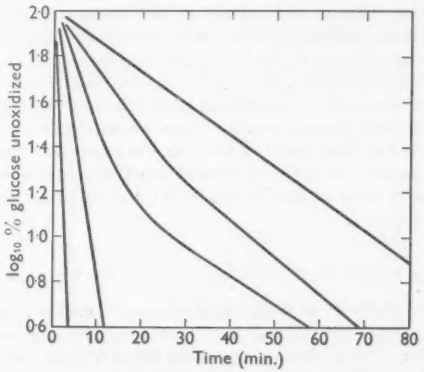


Fig. 3. The oxidation of β-glucose by different quantities of notatin *Y*. The five curves from right to left correspond to 10, 20, 40, 80 and 300 μg. notatin. 0·04 ml. catalase, 3 mg. β-glucose from dangling cup, notatin as indicated, 0·2 M-phosphate (pH 5·6) to 3·3 ml. $T = 20°$. For the rest see explanation of Fig. 2. See also Table 3.

In Table 3 are given the velocity constants K'_β derived from the initial slopes of the curves shown in Fig. 3. The last column of this table shows the activities A_β of this enzyme preparation expressed as the first-order velocity constant K'_β divided by grams dry weight of enzyme. The mean value is 3250.

Table 3. *Relationship between K', the velocity constant for oxidation of β-glucose by notatin Y and the weight of notatin taken*

(Data taken from Fig. 3. Activity of notatin, $A_\beta = K'_\beta$/g. enzyme.)

Notatin (μg./flask)	Initial slope $\left(-\frac{K'_\beta}{2{\cdot}303}\right)$	K'_β	A_β
10	−0·0139	0·0320	3200
20	−0·0268	0·0618	3090
40	−0·0565	0·130	3230
80	−0·120	0·276	3450
300	−0·393	0·905	3020
		Mean	**3250**

The oxidation of α-glucose by notatin Y. Since α-glucose is oxidized very slowly, the analysis of the kinetics of its reaction with notatin can only be carried out in presence of a high concentration of enzyme which simultaneously ensures a reasonably rapid oxidation of the α-form and the virtually instantaneous oxidation of the β-form that appears by mutarotation. In this way the O_2 uptakes can be corrected for the oxidation of β-glucose formed from initially pure α-glucose. Fig. 4 (*b*, *c* and *d*) summarizes the results of experiments carried out in presence of notatin concentrations varying from

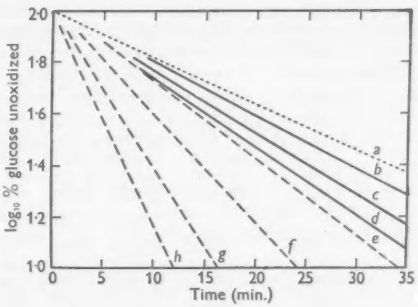


Fig. 4. The oxidation of α-glucose by different concentrations of notatin. 0·04 ml. catalase, 3 mg. α-glucose from dangling cup, 0·2 M-phosphate buffer (pH 5·6) to 3·3 ml. $T = 20°$. Experiments carried out in Barcroft differential manometers in quadruplicate. Full lines (notatin *Y*): *b*, 0·333; *c*, 0·666; and *d*, 0·999 mg. Pecked lines (notatin *Z*): *e*, 0·165; *f*, 0·333; *g*, 0·666; and *h*, 0·999 mg. Dotted line *a*: theoretical line for oxidation β-glucose produced by mutarotation of α-glucose if the latter is not oxidized by notatin.

0·333 to 0·999 mg./3·3 ml. solution in the manometer flasks. In these experiments a small steady-state concentration of β-glucose is rapidly established, after which the rate of the α → β-change and the oxidation of β-glucose become equal. The dotted line (Fig. 4*a*) represents the theoretical course of oxidation of the β-glucose that would be formed by mutarotation of 3 mg. α-glucose on the assumption that α-glucose is not oxidized and that mutarotation is unaffected by the presence of the enzyme preparation. Any increase in the rate of oxidation of α-glucose with increase of notatin concentration can only be ascribed to the catalysis by notatin of either mutarotation, or the direct oxidation of α-glucose, or both. Since, as will be shown later, the sample *Y* (410–417) of notatin does not catalyse mutarotation the increase in the rate of oxidation of α-glucose with notatin concentration must be due to a direct although slow catalytic oxidation of α-glucose.

From the slopes of the lines *b*, *c* and *d* of Fig. 4 the apparent velocity constants K''_α of oxidation of α-glucose by notatin *Y* can easily be calculated (Table 4). The real velocity constant $K'_\alpha = K''_\alpha - k_\alpha$, where $k_\alpha = 0{\cdot}0418$ min.$^{-1}$ is the reaction velocity constant of the forward reaction in the equilibrium

$\alpha \rightleftharpoons \beta$ (see p. 334). The catalytic activity of notatin *Y* in the direct oxidation of α-glucose (A_α) is thus 20·7 (Table 4).

Table 4. *Velocity constants K'_α for oxidation of α-glucose by notatin samples Y and Z and the activities A_α of notatin*

(Data obtained from Fig. 5. The apparent velocity constant $K''_\alpha = K'_\alpha + k_\alpha$, where k_α (=0·0418 min.$^{-1}$) is the velocity constant of the $\alpha \rightarrow \beta$ change in 0·2 M-phosphate buffer pH 5·6 at 20°. Activity of notatin towards α-glucose: $A_\alpha = K'_\alpha$/g. enzyme.)

Sample of notatin	Notatin (mg./3·3 ml.)	Slope $\left(-\frac{K''_\alpha}{2{\cdot}303}\right)$	K''_α	K'_α	A_α
Y	0·333	−0·0212	0·0489	0·0071	21·3
	0·666	−0·0241	0·0555	0·0137	20·6
	0·999	−0·0269	0·0620	0·0202	20·2
				Mean	**20·7**
Z	0·165	−0·0302	0·0695	0·0277	168
	0·333	−0·0421	0·0970	0·0552	166
	0·666	−0·0624	0·1437	0·1019	153
	0·999	−0·0832	0·192	0·150	150
				Mean	**159**

The catalytic activity of notatin *Y* towards α-glucose expressed as a percentage of its activity towards β-glucose is therefore $100 \times 20{\cdot}7/3250 = 0{\cdot}64$ %, which is of the same order as that previously given for mannose and xylose at equilibrium (Keilin & Hartree, 1948*a*).

The effect of substrate concentration upon the rate of reaction at 20° was determined manometrically with 10 μg. notatin *Y* (added from a dangling cup)

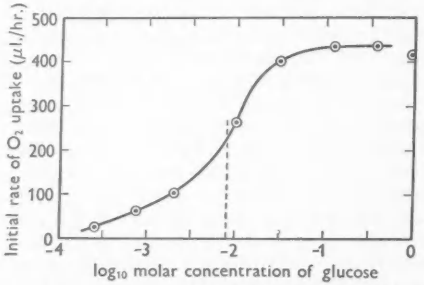


Fig. 5. Determination of Michaelis constant of glucose oxidase at 20°. 10 μg. notatin *Y*, 0·04 ml. catalase. Notatin (in 0·2 ml. buffer) added from dangling cup to catalase + glucose (0·909–0·00025 M) made up to 3·1 ml. with 0·2 M-phosphate buffer pH 5·6.

and glucose concentrations varying from 0·00025 to 0·909 M. From the results thus obtained, which are summarized in Fig. 5, the Michaelis constant K_m is 0·0096 M. It can be argued that because the results were obtained with equilibrium glucose the true K_m for β-glucose is in fact about 64 % of the value given.

The low concentration of glucose that was necessary in the above kinetic experiments (3 mg./flask, or 0·005 M), being less than K_m, lies well within the range where the rate of oxygen uptake is proportional to substrate concentration (Fig. 5). The oxidation of glucose in these experiments is therefore a first-order reaction. Since the true substrate of notatin is β-glucose the above considerations cannot be applied to the much slower oxidation of α-glucose. Although there is no satisfactory method of determining K_m for α-glucose the fact that it does not inhibit the oxidation of β-glucose competitively indicates that α-glucose has a low affinity for notatin and that its oxidation will therefore be a first-order reaction. The same applies to the kinetics of the apparent rate of oxidation of α-glucose depending partly upon mutarotation which is also a first-order reaction.

Oxidation of glucose catalysed by notatin *Z*

The results of manometric experiments on the oxidation of α-, β- and equilibrium glucose catalysed by notatin *Z* are summarized in Fig. 6. These results,

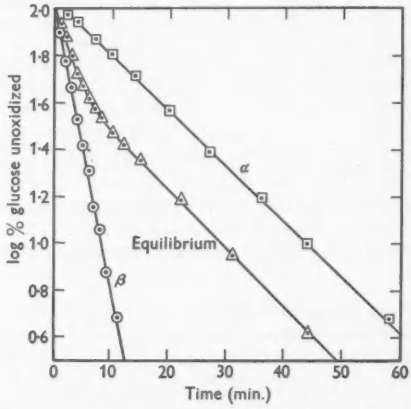


Fig. 6. The oxidation of α-, β- and equilibrium glucose by notatin *Z*. Conditions as in Fig. 2 except that 300 μg. notatin *Z* was used instead of notatin *Y*.

which are very similar to those with notatin *Y* (Fig. 2), clearly show that the enzyme catalyses the oxidation of β-glucose much more rapidly than that of α-glucose. The rate of oxidation of the former is the same with either sample of notatin, but α-glucose is oxidized by notatin *Z* at a rate which is significantly faster than the oxidation by notatin *Y*. This difference is more clearly established by the experiments described in the next paragraph. In the case of equilibrium glucose there is also a break in the curve corresponding to the change from

oxidation of β- to oxidation of α-glucose. This inflexion is reached here within 6 min., the time during which the β-form is removed by oxidation, and therefore the rate of the subsequent oxidation becomes dependent upon the rate of mutarotation $\alpha \rightarrow \beta$ and also upon the rate of oxidation of the α-form.

The oxidation of α-glucose by notatin Z. For this study the concentrations of notatin varied from 0·165 to 0·999 mg./3·3 ml. buffer solution in the additional component which can catalyse either a direct oxidation of α-glucose or its mutarotation to the very easily oxidizable β-glucose. The latter possibility could only be tested polarimetrically.

In order to eliminate the considerable errors that would result from the loss of asymmetry at carbon atom 1 on oxidation of glucose to gluconic acid, the polarimetric measurements were carried out under anaerobic conditions using for this purpose the apparatus shown in Fig. 1. A solution of 9·0 mg. notatin *Y* or *Z* in 18 ml. 0·2 M-phosphate buffer was pipetted into *A* after the required amount of α- or β-glucose had been introduced into *B*. When the sugar had been dissolved anaerobically the mutarotation was followed as previously described.

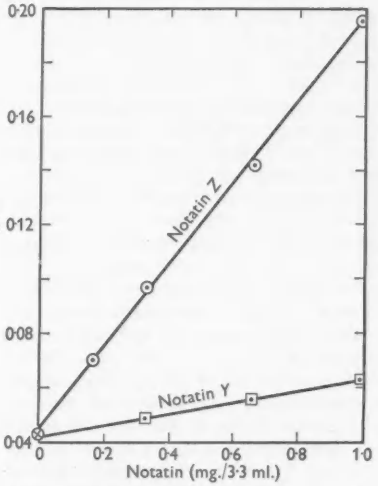


Fig. 7. Relationship between the apparent rate of oxidation of 3 mg. α-glucose expressed as the first-order reaction constants (K''_α) and concentration of notatin *Y* and *Z* (based on data of Fig. 4 and Table 4). Both lines cut the K''_α axis at 0·0418 which is the value of k_α the velocity constant of the change $\alpha \rightarrow \beta$ in absence of notatin.

manometric flasks. The results of these experiments are summarized in Fig. 4. From the slopes of the lines *e*, *f*, *g* and *h* the velocity constants K''_α and K'_α as well as the catalytic activity A_α are calculated as before (Table 4). Thus the enzyme activity (A_α) of notatin *Z* towards α-glucose is about 159 which is eight times higher than the corresponding activity of notatin *Y*. This difference between notatin *Y* and *Z* can be clearly seen in Fig. 7 in which the apparent velocity constants K''_α are plotted against enzyme concentration. In this figure both lines cut the K''_α axis at 0·0418 (k_α).

Polarimetric studies of mutarotation in presence of notatin preparations Y and Z

Although notatins *Y* and *Z* display the same very high catalytic activity in the oxidation of β-glucose, they differ in their catalytic activities towards α-glucose. This suggested that sample *Z* contains an

Table 5. *Mutarotation constants of α- and β-glucose in presence of 0·2 M phosphate buffer,* pH 5·6, *and notatin Y or Z at 20° under anaerobic conditions*

(Notatin concentration 0·5 mg./ml. Experiments carried out in a jacketed 22 cm. polarimeter tube as shown in Fig. 1. S.D. = standard deviation (%).)

Glucose: Isomers	Glucose: Concentration (%, w/v)	Notatin sample	$10^3 K$ (min.$^{-1}$)	S.D.
α	3·98	None	65·8	Data from Table 2
β	4·01	None	66·4	
α	3·99	*Y*	66·2	0·90
β	4·00	*Y*	66·5	0·72
α	3·96	*Z*	131·4	1·00
β	4·01	*Z*	116·5	1·86

The mean values of the mutarotation constants obtained in these experiments (Table 5) show that whereas mutarotation is not influenced by notatin *Y*, it is markedly accelerated by notatin *Z*. These results provide an explanation of the different action of these two samples in the oxidation of α-glucose as shown in Figs. 4 and 7. Moreover, since sample *Y* has no influence on mutarotation, the increasing rates of oxidation of α-glucose by increasing concentrations of notatin (Fig. 4, *b–d*) must be a true measure of the direct oxidation of α-glucose. With notatin *Z* the extra increase in the rate of oxidation of α-glucose is due to the increased mutarotation of α- to β-glucose catalysed by an additional substance present in this sample. Having at their disposal only the notatin sample *Z* ('233–237') Bentley & Neuberger (1949) concluded: 'There is thus suggestive, but not conclusive evidence that notatin catalyses the mutarotation of glucose.' Our experiments, however, have shown that the acceleration of mutarotation is due to a factor distinct from glucose oxidase. The properties of this mutarotation catalyst in its relation to notatin will be examined in a separate paper (Keilin & Hartree, 1951).

Further studies on the specificity of notatin for glucose

Taking the activity of notatin towards β- or equilibrium glucose as 100 the slight degree of oxidation of 6-methyl glucose (1·85) and of 4:6-dimethyl glucose (1·22) together with a few other results previously recorded (Keilin & Hartree, 1948*a*) suggest that it is the 1-, 2- and 3-positions in the glucose molecule which must be free before activation by the enzyme can occur. This is corroborated by our finding that the activity of notatin towards 4:6-benzylidene glucose is 1·90.

Table 6 gives the activity of notatin towards all eight aldohexoses. The various sugars were allowed to equilibrate in solution before being tested, and although the α/β ratios will vary from one sugar to another this can have no serious effect on the overall picture of the high specificity of notatin.

Table 6. *Activity of notatin Y towards* D-*aldohexoses determined manometrically*

(30 mg. monosaccharide; 300 μg. notatin *Y* (only 10 μg. used for oxidation of glucose); 0·04 ml. catalase; 0·2 M-phosphate buffer, pH 5·6; $T = 20°$. Sugar solutions equilibrated overnight before being used.)

Sugar	Activity
Equilibrium glucose (≡β-glucose)	100
Mannose	0·98*
Altrose	0·16
Galactose	0·14*
Talose (two samples)	0·015,† 0
Allose	0
Gulose	0
Idose (syrup)	0·018†

* From Keilin & Hartree (1948*a*).
† These activities cannot be regarded as significant.

It has recently been found by Monod (1950) that improvements in the method of purification of notatin from culture medium filtrates of *P. notatum* yield a product that will not oxidize trehalose and that the slight activity (0·28) that we recorded previously was due to the presence of trehalase. Indeed in the case of trehalose such an explanation is the only possible one since this sugar is a non-reducing one with 1-1′ linkage of the glucose residues. However, from the progress of its oxidation, it was obvious that the oxygen uptake was not due to contamination with glucose. Considering that substitution in the 4- and 6-positions of glucose does not entirely abolish the oxidation by notatin and that with the exception of trehalose the disaccharides tested were either 4- or 6-substituted glucoses, it is conceivable that in some cases at least the observed oxidations of disaccharides were genuine.

THE MOLECULAR WEIGHT OF NOTATIN

According to the published determinations of sedimentation velocity and diffusion constant the molecular weight of notatin is 152,000 (Cecil & Ogston, 1948*a*). Following an investigation into the accuracy of the ultracentrifugal method which involved corrections for errors in cell temperature (Cecil & Ogston, 1948*b*) Dr R. Cecil has re-examined notatin sample *Y* (410–417) and has amended $S_{20} \times 10^{13}$ from 8·27 to 8·09 which corresponds to a molecular weight of 149,000.

DISCUSSION

It is now well established that glucose oxidase or notatin is a chromoprotein enzyme of molecular weight about 149,000 and with two prosthetic groups (alloxazine-adenine dinucleotide) per molecule of protein. In any discussion of the mechanism of the enzyme activity the role of this prosthetic group is of primary importance. All that can be said at present is that the enzyme forms a complex with its highly specific substrate (glucose) which becomes activated. The prosthetic groups acting as hydrogen acceptors are then reduced while glucose undergoes oxidation. In fact the well known anaerobic reduction of flavoprotein enzymes by their substrates has also been demonstrated in the case of notatin (Keilin & Hartree, 1948*a*). In other words, the kinetics of the enzymic oxidation of glucose can be followed spectroscopically under strictly anaerobic conditions. Whether the two atoms of hydrogen are transferred stepwise from glucose to flavine it is difficult to say. Although flavines readily form coloured free radicals by accepting one H (Michaelis, Schubert & Smythe, 1936; Michaelis & Schwarzenbach, 1938), it is unlikely in the case of such an active enzyme as notatin that any intermediate would have an appreciable life.

Once the molecule of glucose is oxidized and the prosthetic group of the enzyme reduced to dihydroflavine, the latter can become reoxidized by a suitable hydrogen acceptor, such as molecular oxygen which becomes reduced to hydrogen peroxide, or under anaerobic conditions, by 2:6-dichlorophenol-indophenol which becomes reduced to a leucoindophenol. In all known cases of oxidation reactions catalysed by autoxidizable flavoprotein enzymes the hydrogen peroxide arises from the reduction of molecular oxygen by the dihydroflavine prosthetic group of the enzyme.

All this shows that glucose oxidase or notatin is a typical aerobic dehydrogenase and this has been confirmed by isotopic experiments carried out in presence of $H_2{}^{18}O$ and $^{18}O_2$ by Bentley & Neuberger (1949). It has long been known that the final product

of the oxidation of glucose catalysed by notatin is gluconic acid. However, Bentley & Neuberger (1949) have demonstrated by estimations of total and free gluconic acid at different stages of the reaction, and by polarimetric experiments, that the first product of oxidation is the neutral δ-gluconolactone which undergoes spontaneous hydrolysis to gluconic acid.

The comparative manometric study of the catalytic activities of notatin in the oxidation of α- and β-glucose required careful correction for mutarotation of α-glucose. An added complication was the presence in some samples of notatin of a catalyst which accelerates mutarotation. It has, however, been possible to eliminate these effects and to establish that the relative rates of oxidation of the two isomers are $\beta : \alpha = 100 : 0{\cdot}64$. In other words, the catalytic activity of notatin in the oxidation of α-glucose is of the same order of magnitude as that previously obtained for some other sugars such as mannose and xylose.

The apparent high activity of notatin towards α-glucose (as high as 77% of that of β-glucose) recorded by Bentley & Neuberger (1949) can only be due to the fact that under their conditions the formation of β-glucose by mutarotation of the α-form proceeded almost as rapidly as the direct oxidation of β-glucose.

The notatin used in our present experiments was taken from two batches labelled '410–417' and '233–237' and referred to in this paper as *Y* and *Z* respectively. Manometric study of the kinetics of oxidation of α- and β-glucose by these samples of notatin gave strong indications of the presence in notatin *Z* but not in notatin *Y* of an additional component capable of catalysing the mutarotation of glucose. This was confirmed by polarimetric measurements carried out under strictly anaerobic conditions. The mutarotating catalyst present only in *Z* increased its apparent activity in the oxidation of α-glucose about eight times and thus raised the rate of its oxidation to about 5% of that of β-glucose.

All eight D-aldohexoses have now been tested with notatin. If its activity towards β- (or equilibrium) glucose = 100, the relative rates for other sugars are: mannose = 0·98, altrose = 0·16, galactose = 0·14, whereas allose, idose, gulose and talose are not oxidized.

In cases where notatin shows an activity towards different sugars one must keep in mind the possibility of the presence in the sample under investigation of glucose as an impurity. This was, for instance, the case with a commercial galactose which in presence of notatin gave a very rapid initial oxygen uptake followed by a slow but steady oxidation of galactose. By extrapolating to zero time we were able to determine that the sample of galactose contained about 4% glucose (Keilin & Hartree, 1948*a*, Fig. 9). It is only when the oxygen uptake shows no sudden change in rate and when it persists over a long period that the sugar can be considered susceptible to direct catalytic oxidation by notatin.

The previous tests with about fifty sugars and their derivatives (Keilin & Hartree, 1948*a*), together with the examination of aldohexoses described in this paper, as well as the fact that the first product of oxidation is δ-gluconolactone (Bentley & Neuberger, 1949), give a clear indication that notatin can be considered as an oxidase highly specific for β-D-glucopyranose.

Every change in configuration and any substitution in the —OH groups either reduces the catalytic activity of notatin to a very low level or abolishes it altogether. So far no compound has been found showing a susceptibility to oxidation by notatin which is intermediate between that of β-glucose (100) and those of 6-methyl glucose (1·85) or 4:6-benzylidene glucose (1·90).

It is perhaps surprising that notatin shows even a slight activity towards α-glucose, considering that stereospecificity is a very general property of enzymes. Inversion at $C_{(2)}$, $C_{(3)}$ or $C_{(4)}$ of β-glucose (to give mannose, allose and galactose respectively) reduces the activity of notatin by at least 99%. But since it is the H atoms associated with $C_{(1)}$ which undergo transfer to the flavine groups of notatin it would be expected that inversion at this point would more effectively interfere with the oxidation.

That an enzyme may form complexes with several substances belonging to the same family as the substrate does not imply that all such substances will undergo activation. The existence of compounds which combine with, but are not activated by, an enzyme is revealed by the competitive inhibition they exercise upon the reaction between the enzyme and its true substrate. Indeed numerous cases of such interesting competitive inhibitors are known for xanthine oxidase, uricase, succinic dehydrogenase and others. The affinity of the enzyme for a competitive inhibitor is sometimes much greater than for the true substrate.

The fact that of the many sugars tested with notatin none has exhibited any appreciable inhibition of oxidation of β-glucose may be taken to indicate that all except β-glucose have either a very small or no affinity for the enzyme. It is conceivable that it is mainly the difficulty in forming a sufficiently stable enzyme-substrate complex that is primarily responsible for the high specificity of the enzyme for β-glucose.

Careful consideration of specificity data recorded in the present and the previous paper (Keilin & Hartree, 1948*a*) gives no clear indication of the

mode of attachment between notatin and its substrate. All that can be said at present is that whereas substitution in the 1-, 2- or 3-OH groups of glucose abolishes enzyme activity entirely, substitution at 4 or 6 or both positions reduces the activity to about 1–2 % of that of β-glucose itself.

In view of the sharp specificity of notatin for the β-form of glucose it becomes important to review the methods we have previously described (Keilin & Hartree, 1948*b*) for the estimation of glucose in terms of oxygen uptake in presence of notatin. When glucose is to be estimated in a static system, i.e. one where glucose is not being formed, the differential rates of oxidation of the two isomers will not affect the final oxygen uptake. As we have already demonstrated, the theoretical uptake is rapidly attained at 39° and must eventually be reached at lower temperatures.

Naturally-occurring derivatives of glucose often have the α-glucopyranose structure (e.g. sucrose, Cori ester) and glucose will be liberated from these in the α-form. There is thus a possibility that the rate of its oxidation may lag behind the rate of its production. At 39° we invariably found that the course of glucose-producing reactions as estimated with notatin could be closely checked through periodic estimations of glucose by independent methods. Even at 20° we found it possible, by using a high concentration of notatin, to obtain a picture of the inversion of sucrose which could be confirmed by the Hagedorn & Jensen procedure. Such agreement may well have been due partly to the presence in the notatin (*Z*) of the component catalysing mutarotation and partly to the high concentration of the oxidase. In fact, even the low activity of notatin towards α-glucose under the above conditions corresponds to a Q_{O_2} of about 300. On the other hand, the experiments that we carried out at 22° on the enzymic hydrolysis of amygdalin and sinigrin presented no complication of this nature, since glucose is present in these glucosides in the β-form and is therefore immediately oxidized by notatin as it is liberated.

Thus the form in which the glucose is liberated will not affect its estimation in terms of oxygen uptake by notatin at 39°. At lower temperatures, however, preliminary manometric experiments must be carried out in order to adjust the concentration of the components of the system, so as to ensure that liberation of glucose and not its oxidation by notatin will limit the rate of the overall reaction.

SUMMARY

1. The catalytic oxidation by notatin of β-glucose is much faster than that of α-glucose.

2. Two samples of notatin (*Y* and *Z*), which were of equal catalytic activity in the oxidation of β-glucose, showed very different activities in the oxidation of α-glucose. Sample *Z* oxidized α-glucose about eight times faster than sample *Y*.

3. Manometric study of the oxidation of α-glucose by these samples of notatin indicated that sample *Z* contained an additional component capable of catalysing mutarotation.

4. This was confirmed by polarimetric study of the effect of the two samples of notatin on mutarotation of glucose under strictly anaerobic conditions, the experiments being carried out in an apparatus specially devised for this purpose.

5. Making use of notatin *Y*, which did not influence mutarotation, the rates of the direct oxidations of α- and β-glucose were obtained by correcting the manometrically observed rates of oxygen uptake for the calculated mutarotation of the α- to the easily oxidizable β-form under the influence of the buffer solution. The ratio of oxidation rates thus obtained is $\beta : \alpha = 100 : 0{\cdot}64$ at 20°.

6. The bearing of this result on the use of notatin for estimation of glucose formation in biological systems is discussed. The oxygen uptake in presence of notatin is a reliable measure of glucose formation at any temperature if glucose is liberated in the β-form, while at 39° the mutarotation is fast enough to ensure that the oxygen uptake is a true measure of the rate of appearance of either isomer. At lower temperatures, if glucose is liberated in the α-form the concentration of notatin must be carefully adjusted so that the oxidation of glucose does not limit the rate of the overall reaction.

7. All eight D-aldohexoses have been tested with notatin. If the rate of oxidation of β-glucose = 100, those of the other sugars are: mannose = 0·98, altrose = 0·16 and galactose = 0·14, while allose, gulose, idose and talose are not oxidized.

8. The high specificity of notatin for β-glucose is indicated by the fact that optical inversion at any carbon atom or substitution in the hydroxyl groups of the substrate either reduces the catalytic activity of notatin to about 1 % of its original value or abolishes it altogether.

9. Notatin can now be considered as a specific β-D-glucopyranose oxidase.

We wish to thank Dr A. Gottschalk and Dr D. J. Bell for much valuable advice, and Dr R. Cecil for re-examining notatin in the ultracentrifuge. We are greatly indebted to Prof. C. S. Hudson, Prof. T. Reichstein and Dr H. S. Isbell for their gifts of rare monosaccharides.

REFERENCES

Bentley, R. & Neuberger, A. (1949). *Biochem. J.* **45**, 584.
Campbell, P. N. & Creasy, N. H. (1949). *Biochem. J.* **44**, xl.
Cecil, R. & Ogston, A. G. (1948*a*). *Biochem. J.* **42**, 229.
Cecil, R. & Ogston, A. G. (1948*b*). *Biochem. J.* **43**, 592.
Cori, G. T. & Cori, C. F. (1940). *J. biol. Chem.* **135**, 733.
Coulthard, C. E., Michaelis, R., Short, W. F., Sykes, G., Skrimshire, G. E. H., Standfast, A. F. B., Birkinshaw, J. H. & Raistrick, H. (1942). *Nature, Lond.*, **150**, 634.
Coulthard, C. E., Michaelis, R., Short, W. F., Sykes, G., Skrimshire, G. E. H., Standfast, A. F. B., Birkinshaw, J. H. & Raistrick, H. (1945). *Biochem. J.* **39**, 24.
Hudson, C. S. & Dale, J. K. (1917). *J. Amer. chem. Soc.* **39**, 323.
Hudson, C. S. & Yanovsky, E. (1917). *J. Amer. chem. Soc.* **39**, 1035.
Isbell, H. S. & Pigman, W. W. (1933). *Bur. Stand. J. Res., Wash.*, **10**, 337.
Isbell, H. S. & Pigman, W. W. (1937). *Bur. Stand. J. Res., Wash.*, **18**, 141.
Keilin, D. & Hartree, E. F. (1945). *Biochem. J.* **39**, 148.
Keilin, D. & Hartree, E. F. (1946). *Nature, Lond.*, **157**, 801.
Keilin, D. & Hartree, E. F. (1948*a*). *Biochem. J.* **42**, 221.
Keilin, D. & Hartree, E. F. (1948*b*). *Biochem. J.* **42**, 230.
Keilin, D. & Hartree, E. F. (1952). *Biochem. J.* **50**, 341.
Michaelis, L., Schubert, M. P. & Smythe, C. V. (1936). *J. biol. Chem.* **116**, 587.
Michaelis, L. & Schwarzenbach, C. (1938). *J. biol. Chem.* **123**, 527.
Monod, J. (1950). Private communication.
Müller, D. (1928). *Biochem. Z.* **199**, 136.
Müller, D. (1941). *Enzymologia*, **10**, 40.
Reeve, K. D. (1951). *J. chem. Soc.* p. 172.
Richards, E. M., Faulkner, I. J. & Lowry, T. M. (1927). *J. chem. Soc.* p. 1733.
Zerfas, L. (1931). *Ber. dtsch. chem. Ges.* **64**, 2289.

Biological Catalysis of Mutarotation of Glucose

By D. KEILIN and E. F. HARTREE
Molteno Institute, University of Cambridge

(*Received* 23 *May* 1951)

We have shown in previous papers (Keilin & Hartree, 1948*a*, 1952) that glucose oxidase, or notatin, is highly specific for β-glucose, the rate of oxidation of α-glucose being only about 0·64 % of that of β-glucose.

While determining the relative rates of oxidation of α- and β-glucose by two purified samples of notatin (*Y* and *Z*) we found that although their activities towards β-glucose, or equilibrium glucose, were virtually the same, their activities towards α-glucose differed very markedly, notatin *Z* being in this respect about eight times more active than notatin *Y*. This difference was ascribed by us to the presence in sample *Z* of an additional component capable of accelerating mutarotation.

In the present paper we shall examine the properties of this component and compare them with those of notatin itself. This will enable us to bring forward fresh evidence in support of the view that the mutarotation accelerating substance is independent of notatin, although until now the greatest concentration of this substance has been found in some highly purified samples of notatin. On the other hand, no attempt to isolate it in the pure form could be made without sacrificing much of the notatin which we could scarcely afford, considering the very limited availability of this enzyme and the great demand upon it for the study of kinetics of reactions catalysed by different enzymes (Keilin & Hartree, 1948*b*).

Since the properties of the substance accelerating mutarotation are those of a highly active, thermolabile, non-dialysable catalyst we propose for it the name *mutarotase*. Considering that a variety of substances are capable of catalysing mutarotation, and that the intimate mechanism of such catalysis is still unknown, the name mutarotase given to the catalyst found in notatin preparations can only be regarded as a convenient provisional term, pending further evidence as to its enzymic nature. This would require more information on the properties of this catalyst based upon the study of preparations which are more active and more readily available than the highly purified samples of notatin used in the present study.

MATERIALS AND METHODS

Notatin. The two samples *Y* and *Z* described in the previous paper (Keilin & Hartree, 1952) were used in the present study.

Catalase, α- and β-glucose and phosphate buffer were the same materials as used in the previous paper.

α-Methyl glucoside had been prepared by the standard procedure from glucose and methanolic HCl.

β-Methyl glucoside was prepared by the method of Müller (1931). The melting point and mixed melting point with an authentic sample were 107–109° (uncorr.).

Fructose, lactose, maltose, xylose and sorbitol were pure bacteriological grades.

Anaerobic polarimetric measurements of mutarotation and manometric estimations of glucose oxidase activity were carried out as described in our previous paper.

RESULTS

It has already been established (Keilin & Hartree, 1952) that the addition under anaerobic conditions of 0·5 mg./ml. of notatin *Z* to a 4% solution of either α- or β-glucose in 0·2M-phosphate buffer pH 5·6, at 20°, approximately doubles the mutarotation constant (*K*).

Effect of enzyme concentration on mutarotation

In Table 1 and Fig. 1 are summarized the results of further polarimetric experiments carried out anaerobically, as previously described, on the effect of varying concentrations of notatin *Z* on the mutarotation of α- and β-glucose. These experiments show that the increment of the mutarotation constant above the value for buffer is proportional to notatin concentration and therefore to the concentration of mutarotase. Thus if the mutarotation constants are plotted against notatin concentrations a straight line is obtained which cuts the *K* axis at 0·0664, the mutarotation constant in 0·2M-phosphate buffer, pH 5·6. These experiments also show that *K* is significantly higher for α- than for β-glucose. Moreover, the values of *K* calculated from successive readings of optical rotation slightly drifted with time: upward for β-glucose and downward for α-glucose. The constant thus tended towards a common mean as the mutarotation proceeded. However, since the equilibrium values of rotation were not influenced by the enzyme preparation the accelerating effect was a true catalysis.

Table 1. *Effect of different concentrations of notatin Z ('233–237') on the mutarotation of α- and β-glucose in presence of 0·2M-phosphate buffer, pH 5·6, at 20°*

(S.D.: standard deviation. Data obtained by polarimetric measurements carried out anaerobically.)

Glucose				
Isomer	Concentration (%, w/v)	Notatin concentration (mg./ml.)	$10^3 K$ (min.$^{-1}$)	S.D. (%)
α	4·0	1·0	185·9	1·24
β	4·0	1·0	168·7	2·61
α	4·0	0·5	131·4	1·00
β	4·0	0·5	116·5	1·86
α	4·0	0·3	101·0	1·58
β	4·0	0·3	96·7	0·96
α	4·0	0·2	90·7	1·40
β	4·0	0·2	85·0	1·41
α	4·0	0·1	78·6	1·44
β	4·0	0·1	75·6	1·25
α	4·0	0·0	65·8*	0·84
β	4·0	0·0	66·4*	1·11

* Values in absence of notatin taken from the previous paper (Keilin & Hartree, 1952).

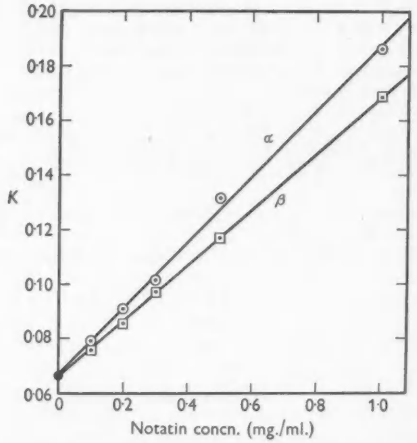


Fig. 1. The influence of the concentration of notatin *Z* on the mutarotation constant for α- and β-glucose (4%, w/v) in presence of 0·2M-phosphate buffer, pH 5·6, at 20°. ● = Mutarotation constant in buffer.

The effect of glucose concentration on the enzyme-catalysed mutarotation

The experiments carried out with different concentrations of α- and β-glucose, which are summarized in Table 2 and Fig. 2, show that the mutarotation constant falls with increasing glucose concentration and approaches the value of *K* for buffer alone (which is of course independent of glucose concentration). This unexpected relationship between *K* and substrate concentration will be discussed later.

Effect of other sugars on the catalysis of mutarotation and on the oxidation of glucose

The effect of various sugars on the mutarotation of α-glucose in presence of notatin *Z* was tested under the usual anaerobic conditions except that the notatin (0·27 ml. of 2% aqueous solution) was kept separate from the solution of the second sugar until all oxygen had been removed from the apparatus. This was achieved by placing the notatin solution in a small side bulb (Keilin & Hartree, 1952, Fig. 1, *H*). α-Glucose (400 ± 1 mg.) was weighed into the apparatus while the second sugar (4 mol. proportions) was dissolved in phosphate buffer (18 ml.) and allowed to stand overnight to reach rotational equilibrium before being placed

in the apparatus. In every case a control experiment in absence of notatin was carried out. From the results of these experiments, which are summarized in Table 3, it may be seen that the greatest inhibition is obtained with sugars which have a close

Table 2. *Effect of different concentrations of glucose on mutarotation of α- and β-glucose in presence of notatin Z and 0·2M-phosphate buffer*, pH 5·6 *at* 20°

(Data obtained polarimetrically under anaerobic conditions.)

Glucose				
Isomer	Concentration (%, w/v)	Notatin concentration (mg./ml.)	$10^3 K$ (min.$^{-1}$)	S.D. (%)
α	1·33	0·2	125·5	1·68
β	1·33	0·2	117·5	2·81
α	2·66	0·2	98·3	1·47
β	2·66	0·2	94·0	1·03
α	4·00	0·2	90·7	1·40
β	4·00	0·2	85·0	1·41
α	5·65	0·2	82·2	0·94
β	5·65	0·2	80·1	0·65
α	4·00	0·0	65·8*	0·84
β	4·00	0·0	66·4*	1·11

* Values in absence of notatin taken from Keilin & Hartree (1952).

structural resemblance to glucose and, in particular, to β-glucose; in fact xylose and β-methyl glucoside are at least as effective as an excess of equilibrium glucose. Since the relative proportions of the α- and β-forms of the different sugars will vary, no strictly quantitative significance can be ascribed to the values expressing the relative degrees of inhibition of mutarotation produced by these sugars (Table 3). Owing to the very limited amount of notatin available no attempt was made to determine whether these inhibitions were competitive.

The effects of the same sugars on the rate of oxidation of glucose by glucose oxidase were also examined. In these experiments, which were carried out manometrically, the right-hand flasks received 30 μg. notatin, catalase, 0·3 ml. 10% equilibrium glucose (in dangling tubes) and ten equivalents of the second sugar. The left-hand flasks received everything except the equilibrium glucose so as to compensate for any slow oxidation of the second sugar. The very slight inhibition shown in a few instances (Table 3) has no bearing upon the structural relationship between the substrate and the inhibitor.

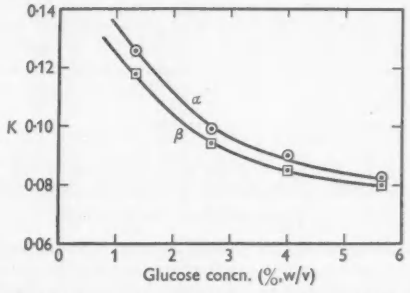


Fig. 2. The influence of the concentration of α- and β-glucose on the mutarotation constants in a solution of notatin Z (0·2 mg./ml.) in 0·2M-phosphate buffer, pH 5·6, at 20°.

Effect of heating notatin Z on the catalysis of mutarotation

For the study of thermolability of mutarotase a solution of notatin Z containing 0·5 mg./ml. phosphate buffer was heated, in 20 ml. portions, under the varying conditions shown in Table 4. The different samples were then tested for their effect on mutarotation. For these tests, to 18 ml. of the

Table 3. *Effects of a second sugar (a) on the mutarotation under anaerobic conditions of α-glucose in presence and in absence of notatin Z; and (b) on the oxidation of equilibrium glucose by notatin Z*

((*a*) 400 mg. α-glucose + 4 equiv. of second sugar in 18 ml. 0·2M-phosphate buffer, pH 5·6; notatin 0·3 mg./ml.; 20°. (*b*) Oxidation of glucose determined manometrically: 30 mg. equilibrium glucose and 10 equiv. of second sugar; 30 μg. notatin; 0·04 ml. catalase; 20°.)

	$10^3 K$ (mutarotation)		Inhibition by second sugar in presence of notatin (%)	
Second sugar	Without notatin	With notatin	Mutarotation of α-glucose	Oxidation of equilibrium glucose
None	66·7	129·2	—	—
β-Methyl glucoside	66·1	75·0	86	3
Xylose	63·7	73·9	83	8
Equilibrium glucose	64·6	77·8	78	0
Maltose	66·9	88·8	65	15
α-Methyl glucoside	67·5	90·5	63·5	0
Sorbitol	62·6	96·0	46·5	0
Lactose	64·6	105·3	35·0	12
Fructose	62·2	106·1	29·8	0

heated and filtered notatin solution, α-glucose was added anaerobically to give a concentration of 4%. The results of these experiments, summarized in Table 4, show that heating at 65° for only 10 min. abolishes the mutarotating activity of the enzyme preparation by 86%.

Table 4. *Irreversible inactivation of mutarotase after heating notatin to different temperatures*

(Mutarotation experiments carried out under the usual anaerobic conditions with 4% α-glucose and notatin *Z* (0·5 mg./ml.) at 20°.)

Treatment of notatin	$10^3 K$ (min.$^{-1}$)	Inactivation (%)
Untreated	131·4	—
60° for 10 min.	118·4	20
65° for 10 min.	75·2	86
80° for 10 min.	72·6	90
100° for 30 min.	67·2	99
Control (no notatin)	66·4	—

Table 5. *The effect of* 20 *min. treatment at* pH 9·1 *followed by neutralization to* pH 5·6 *on mutarotation and on rate of glucose oxidation by notatin Z*

(For mutarotation, 720 mg. α-glucose was dissolved anaerobically in 18 ml. phosphate-borate buffer without and with notatin (final concentration 0·5 mg./ml.). Oxidase activity was determined as in Table 3 using 30 mg. equilibrium glucose and 0·1 ml. of the same phosphate-borate-notatin solution; temperature 20°. Standard deviations are absolute values and not percentages as given elsewhere.)

Solutions used for teste	$10^3 K$ (min.$^{-1}$)	O_2 uptake (μl./hr.) (based on initial rate)
(I) Notatin incubated at pH 9·1 and neutralized to pH 5·6	105·4±6·1*	174
(II) Notatin buffered to pH 5·6	110·0±1·3	1380
(III) Phosphate-borate buffer as used for I and II	53·6±0·73	—

* The high standard deviation was due to difficulties in taking readings with a slightly cloudy solution.

The effect of prior incubation at pH 9·1 *on the mutarotase and glucose oxidase activities of notatin Z*

For these experiments the following solutions were prepared: (*a*) notatin *Z*, 5 mg./ml. in water; (*b*) 0·05 M-borax; (*c*) phosphoric acid, approximately 1 M; (*d*) 0·2 M-phosphate buffer, pH 5·6; (*e*) a mixture of 20 ml. borax and 2·0 ml. phosphoric acid (pH 5·6). From these solutions were prepared three mixtures as follows:

(I) 2 ml. *a* was added to 8 ml. *b*. After 20 min. at 20° the solution was neutralized to pH 5·6 by the addition of 9·2 ml. *d* and 0·8 ml. *c*.

(II) 2 ml. *a* was added to a mixture of 9·2 ml. *d* and 8·8 ml. *c*.

(III) As (II), but the notatin solution (*a*) was replaced by water.

In (I) the notatin had been at pH 9·1 for 20 min. while in (II) the pH had been maintained at 5·6. The inorganic constituents were identical in all three mixtures. To estimate glucose oxidase activity 0·1 ml. each of (I) and (II) were tested manometrically with catalase and 30 mg. equilibrium glucose under the standard conditions. For the determination of mutarotase activity, 18 ml. of (I), (II) and (III) were examined under anaerobic conditions in the polarimeter with 720 mg. α-glucose.

From the results of these experiments (Table 5) it is seen that while the high pH has inactivated the glucose oxidase by 87%, the mutarotase activity is not significantly affected.

Fractionation of notatin Z

Further confirmation of the view that mutarotation and oxidation of glucose by notatin *Z* are catalysed by two distinct enzymes, mutarotase and glucose oxidase, was obtained by a simple fractionation procedure.

For this purpose a 0·2% (w/v) aqueous solution of notatin *Z* was prepared and the insoluble residue filtered off. Of this solution (130 ml.), now containing 1·84 mg./ml., 5 ml. was set aside as a control. To the remaining 125 ml. was added 56·9 g. $(NH_4)_2SO_4$ to give 0·70 saturation. The resulting precipitate was centrifuged off, washed with 0·70 saturated $(NH_4)_2SO_4$, dissolved in a little water and dialysed against distilled water until salt free (fraction *A*). Two further fractions (*B* and *C*) were similarly obtained by raising the $(NH_4)_2SO_4$ concentration successively to 0·80 and 0·90 saturation respectively.

To 16 ml. *A* was added 1 ml. tricalcium phosphate gel (35·4 mg. dry weight). The mixture, which had a pH of 6·8, was centrifuged to give a clear pale-yellow solution and a yellow residue. The former was dialysed as before to give fraction *A* i. The yellow residue was washed with 5 ml. distilled water, the water was discarded and the residue resuspended in 2 ml. water. The adsorbed material was removed from the calcium phosphate by addition of 0·5 ml. saturated potassium hydrogen oxalate which brought the pH to 7·4. The mixture was centrifuged and the clear bright-yellow eluate poured off. The almost white residue was again extracted with 3 ml. water and the extract added to the yellow eluate. This solution was dialysed to give fraction *A* ii.

The volumes, dry weights and activities of the various fractions are given in Table 6. The activities are expressed as the initial rates of O_2 uptake obtained under the standard conditions but with 3·0 mg. equilibrium glucose. The volumes of enzyme taken for the activity determination were adjusted so that the rate of uptake did not exceed 10 μl./min. since under these conditions the rate is constant for at least 5 min. and is directly proportional to the enzyme concentration. From the activity figures the volume of each fraction containing the same quantity of glucose oxidase as 0·2 ml. of the control could easily be calculated (Table 6, column 6).

In Fig. 3 are shown the apparent rates of oxidation by equivalent amounts of the various fractions,

Table 6. *Activities of glucose oxidase solutions obtained by fractionating notatin Z*

(Glucose oxidase activities expressed as μl. O_2 uptake/min./0·01 ml. enzyme solution in presence of 0·3 ml. 1% equilibrium glucose and 0·04 ml. catalase in 0·2M-phosphate buffer pH 5·6 at 20°.)

Fractions	Colour	Vol. (ml.)	Dry wt. (mg./ml.)	Activity	Volumes containing equivalent quantities of enzyme (ml.)
Notatin *Z*	Pale yellow	—	1·84	7·10	[0·2]
A	Yellow	26·5	2·15	14·67	0·0965
B	Yellow	13·5	3·95	19·22	0·0734
C	Yellow-brown	3·0	1·37	6·63	0·214
*A*i	Yellow	16·0	2·60	13·62	0·0938
*A*ii	Pale yellow	7·3	0·78	4·52	0·314

of 3 mg. α-glucose. The results are plotted logarithmically as in the preceding paper (Keilin & Hartree, 1952). The dotted line in this figure represents the theoretical course of the $\alpha \rightarrow \beta$ change in

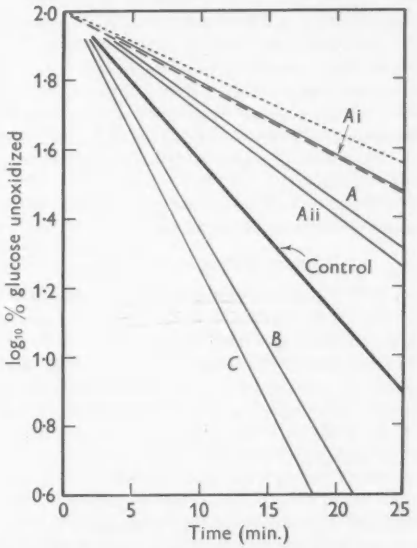


Fig. 3. Apparent rates of oxidation of 3 mg. α-glucose (plotted logarithmically) catalysed by various fractions obtained from notatin *Z* (see Table 6). Experiments carried out in presence of 0·04 ml. catalase, 0·2M-phosphate buffer, pH 5·6, at 20°; enzyme quantities as shown in Table 6 (column 6); - - - - - -, theoretical course (if α-glucose would not be oxidized directly) depending upon mutarotation due to phosphate buffer; — — —, expected course if α-glucose is oxidized directly in absence of mutarotase.

phosphate buffer, if the back reaction were abolished, and hence the rate of oxidation if α-glucose were not directly oxidized by notatin. However, a slow direct oxidation of α-glucose by notatin does take place and it can be calculated from our previous results (Keilin & Hartree, 1952, fig. 7) that with the glucose oxidase present in 0·2 ml. of control solution, the reaction would proceed as shown by the pecked line.

The increase in slopes of the full lines in Fig. 3, as compared with the pecked line, is thus a measure of the mutarotase/glucose oxidase ratios of the various fractions.

Since the oxidase activity of all fractions is the same, they must differ markedly in their content of mutarotase, which in fractions *B* and *C* was much higher, and in *A* and *A*ii much lower, than in the original preparation. Fraction *A*i from notatin *Z* was completely devoid of mutarotase, and thus resembled in this respect the notatin *Y* examined in the previous paper (Keilin & Hartree, 1952).

Detection of mutarotase in culture medium of Penicillium notatum

The study of mutarotase in culture medium was carried out manometrically. The strain of *P. notatum* used by Coulthard *et al.* (1945) for the preparation of notatin was grown at 24° on the medium *A* of these workers. Cultures were kindly supplied by Mr C. E. Coulthard of the Boots Pure Drug Company.

After 1 week the medium was collected and filtered, 100 ml. lots were frozen at −15° in thin layers and dried, in the frozen state, in a vacuum desiccator (0·1 mm. Hg) over H_2SO_4 and KOH. The resulting light-brown powder was dissolved in about 10 ml. water and dialysed for 24 hr. against distilled water at 5°. The solution (15 ml.) was again dried in the frozen state and the residue dissolved in 5 ml. water (concentrate *C*). The ratio of the rates of oxidation of α- and β-glucose was obtained as follows: under the standard conditions for manometric experiments described in the previous paper (Keilin & Hartree, 1952), the complete oxidations of 3·0 mg. α-glucose by (1) 300 μg. notatin *Y* (which is devoid of mutarotase) and (2) 300 μg. *Y* plus 0·5 ml. *C*, were followed (Fig. 4, *a* and *b*). An experiment identical with (2) except that heated *C* was used showed that no thermostable catalysts of mutarotation, such as salts, were present in *C* (*c*). Two further experiments

(*d* and *e*) compared the rates of oxidation of 3·0 mg. α- and β-glucose by 0·5 ml. *C*.

The first-order reaction constants ($-2{\cdot}30 \times$ slope), as given in Fig. 4, were defined in the previous paper: K''_α (measured constant for α-glucose) $= K'_\alpha + k_\alpha$, where K'_α = sum of the constants for the direct oxidation of α-glucose and for the enzyme-catalysed mutarotation $\alpha \rightarrow \beta$ and k_α = constant for $\alpha \rightarrow \beta$ change in the phosphate buffer.

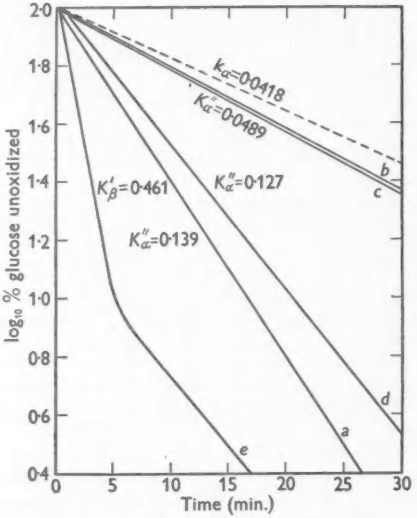


Fig. 4. Oxidation of 3·0 mg. β-glucose and apparent oxidation of 3·0 mg. α-glucose (plotted logarithmically) by a freeze-dried concentrate *C* (0·5 ml.) of a 7-day culture filtrate of *P. notatum*. Conditions as for Fig. 3. *a*, *C* + 300 μg. notatin *Y* + α-glucose; *b*, notatin *Y* + α-glucose; *c*, boiled *C* + notatin *Y* + α-glucose; *d*, *C* + α-glucose; *e*, *C* + β-glucose. – – –, theoretical course (if α-glucose would not be oxidized directly) depending upon mutarotation due to phosphate buffer.

The value of K'_α for 0·5 ml. *C*, i.e. the difference between the constants obtained from experiments *a* and *b*, is 0·090. In these two experiments the presence of excess notatin *Y* ensures that β-glucose, as it is formed, is oxidized virtually instantaneously and that, in consequence, the difference is a true measure of the sum of the oxidation of α-glucose by *C* and any catalysis of mutarotation additional to the buffer contribution. If *C* contains sufficient glucose oxidase to oxidize the β-glucose as rapidly as it is formed from the α-form, then a true value of K'_α could also be obtained from the difference between the constant for *d* (oxidation of α by *C*) and the constant for the $\alpha \rightarrow \beta$ change (pecked line in Fig. 4). Since the value of K'_α obtained in this way is very close to that obtained from *a* and *b* it follows that 0·5 ml. *C* does in fact contain almost sufficient glucose oxidase.

Experiment *e* gives directly the value of K'_β (0·461). In the preceding paper (Keilin & Hartree, 1952; Table 3) the activity of notatin towards β-glucose was defined as $A_\beta = \frac{K'_\beta}{\text{g. enzyme}}$ and a value of 3250 was obtained for an enzyme preparation which was about 90 % pure. From these figures it can be calculated that 0·5 ml. *C* contains about 130 μg. of glucose oxidase. Since the dry weight of 0·5 ml. *C* is 5·0 mg., the preparation is about 2·6 % pure with respect to glucose oxidase. The change of slope in line *e* itself indicates a high mutarotase activity. In the preceding paper (Keilin & Hartree, 1952; fig. 3), this inflexion was observed when using 20 or 40 μg. notatin in absence of mutarotase and it appeared to be due to the $\beta \rightarrow \alpha$ change during the first part of the experiment. The β-glucose was thus exhausted before the oxygen equivalent to 3·0 mg. glucose had been taken up and the latter part of the oxidation was limited by the rate of the $\alpha \rightarrow \beta$ back reaction in the phosphate buffer. With 80 μg. notatin (≡ about 72 μg. pure enzyme) the oxidation was complete before an appreciable degree of mutarotation could take place. In the present experiments, however, the increased rate of mutarotation can affect the course of the oxidation of β-glucose by as much as 130 μg. glucose oxidase.

For *C* the rate of oxidation of α-glucose, as percentage of the rate for β-glucose is thus

$$0{\cdot}090/0{\cdot}461 \times 100,$$

i.e. ~ 20 %. The comparable figures for mutarotase-free glucose oxidase is 0·64 % and for notatin *Z* ~ 5 % (Keilin & Hartree, 1952).

A second set of experiments similar to the above was carried out with a culture medium concentrate prepared in a slightly different manner: the filtered medium (100 ml.) was dialysed against distilled water until salt-free (24 hr.), and then freeze-dried and redissolved in 5 ml. water. Using this material it was found that both K''_α for α-glucose + concentrate, and K'_β (corresponding to *d* and *e* in Fig. 4) were reduced to about 0·049. Since the direct oxidation of β-glucose is a measure of glucose oxidase activity it follows that this enzyme had been approximately 90 % destroyed by the second method of concentration. However, K''_α for α-glucose + concentrate + notatin *Y* (cf. *a*) remained unchanged, indicating that the activity of mutarotase was the same in both concentrates.

Mutarotase in mycelia of P. notatum

Two 7-day mycelia (15 cm. diam.), grown as described in the previous section, were well washed with water, dried between filter paper, and disintegrated under 250 ml. acetone in a Waring blender. The mixture was filtered on a Büchner funnel and the powder allowed to dry in the air.

The powder (2·4 g.) was extracted for 30 min. with 72 ml. 0·2M-phosphate buffer pH 5·6 and centrifuged. The pale-yellow supernatant fluid was clarified by filtration through a Whatman no. 3 paper.

Manometric studies of mutarotation in this extract were not possible owing to the presence of an inhibitor of glucose oxidase. Tests for mutarotase were therefore carried out by the anaerobic polarimetric method described in the previous paper (Keilin & Hartree, 1952). The mutarotation constant K was determined using 4% (w/v) α-glucose in (*a*) 0·2M-phosphate buffer, pH 5·6, and (*b*) the buffer extract of the acetone powder. K was found to be 28% higher with *b* than with *a*.

DISCUSSION

We have previously shown that some samples of purified notatin, in addition to their highly specific catalytic activity in the oxidation of β-glucose, also have the property of accelerating the mutarotation of glucose. This property was found to be due to the presence in some samples of a mutarotation catalyst which, being thermolabile and not dialysable, is described here under the name of *mutarotase*.

Mutarotase and glucose oxidase. The evidence that mutarotase is not identical with glucose oxidase itself can briefly be summarized as follows:

(1) The two catalysts are not necessarily present in the same samples of notatin; thus sample *Y* contains only glucose oxidase, whereas sample *Z* contains both.

(2) By treatment of notatin *Z* with ammonium sulphate and calcium phosphate several fractions were obtained, one of which, like notatin *Y*, was completely devoid of mutarotase, whereas others showed mutarotase/oxidase ratios more than double that of notatin *Z*.

(3) A concentrated culture medium filtrate of *P. notatum* showed a mutarotase/oxidase ratio four times higher than that of notatin *Z*. A modification in the method of concentration reduced the glucose oxidase activity by about 90% without affecting the mutarotase. Such a preparation oxidized α- and β-glucose at essentially the same rate.

(4) The two catalysts show very marked differences in their resistance to alkali. Thus, treatment for 20 min. at pH 9·1 irreversibly destroys the glucose oxidase activity of notatin by nearly 90% but mutarotase activity is scarcely affected.

(5) They show very different susceptibilities to the action of other sugars; whereas glucose oxidase activity, on addition of ten equivalents of other sugars, is either not affected at all or is inhibited by not more than 15%, mutarotase activity, in presence of four equivalents of these sugars, is inhibited from 30 to 90%.

(6) They show an entirely different behaviour in relation to substrate concentration; whereas the activity of glucose oxidase is proportional to glucose concentration until the enzyme approaches saturation with its substrate, the activity of mutarotase is approximately proportional to the reciprocal of the glucose concentration.

That the mutarotation catalyst is not an artifact, or a derivative of glucose oxidase produced by the treatment involved in the purification of the enzyme, is clearly shown by the fact that mutarotase can be detected in the culture medium and can be extracted, together with glucose oxidase, from the acetone-dried mycelium of *P. notatum*.

Reaction kinetics of mutarotase. In the experiments summarized in Tables 1 and 2 the calculated first-order reaction constants were in fact constant during the run except for the very slight upward and downward drifts with time in the case of β- and α-glucose respectively. This means that the mutarotation catalysed by mutarotase, apart from the buffer contribution, is in fact a first-order reaction in respect to glucose. The enzyme is thus not saturated with its substrate within the experimental range of glucose concentration and, if the enzyme concentration is fixed, K should be independent of glucose concentration within this range. The experiments recorded in Table 2 and Fig. 2, however, clearly show an inverse relationship between the mutarotation constant and glucose concentration, the rate of mutarotation falling off with increase of glucose concentration and gradually approaching that occurring in the phosphate buffer alone. Although the inhibition of enzyme activity by excessive concentrations of substrate has often been recorded, no explanation of this phenomenon has yet been given, which would apply equally well to all instances. In the case of mutarotation the inhibition by extra molecules of glucose is very similar to that obtained with structurally similar sugars or glucosides. Moreover, since β-methyl glucoside is more effective than α-methyl glucoside it is reasonable to assume that, as an inhibitor, β-glucose is more effective than the α-form. This would explain the fact that α-glucose invariably yielded higher values of K than did β-glucose (Tables 1 and 2).

Activity of mutarotase. The fact that much higher concentrations of notatin *Z* are necessary for demonstration of mutarotase activity than for oxidation of glucose does not imply that mutarotase is not highly active. In the first place the molar concentration of notatin in the mutarotation experiments was very much lower than that of the inorganic catalysts of mutarotation (water and ions), and in the second place not more than a small fraction of notatin *Z* consists of mutarotase. This latter point is a reasonable deduction from the diagrams of the sedimenting boundaries of notatin *Z* given by Cecil & Ogston (1948). From these diagrams it is clear

that the major impurity could not account for much more than 10% of the total sedimenting material and that as the sedimentation velocity of this impurity is about half that of glucose oxidase, its molecular weight will be approximately half that of notatin, i.e. about 75,000.

In presence of 4% glucose the value of K in the 0·2M-phosphate buffer is $6{\cdot}6 \times 10^{-2}$, while for a solution of notatin Z alone (1 mg./ml.) it is $11{\cdot}1 \times 10^{-2}$; hence the 'catalytic coefficient' of mutarotase, i.e. the velocity constant for a molar solution, would be $11{\cdot}1\text{M}/x$, where x is the percentage mutarotase in notatin Z (in terms of the dry weight). On the other hand, with 1·33% glucose the catalytic coefficient would be $26\text{M}/x$ (Table 2), while still higher values would presumably be attainable at lower glucose concentrations.

If it is assumed that the major impurity in notatin Z is in fact mutarotase then the above figures, 75,000 and 10, can be substituted for M and x to give an approximate estimate of the catalytic coefficient of mutarotase. The value so obtained is of the order of 2×10^5 min.$^{-1}$ which is significantly higher than the figure for the OH^- ion ($8{\cdot}8 \times 10^3$), the most active catalyst for mutarotation so far recorded (Smith, 1936; Smith & Smith, 1937). On the other hand, should mutarotase be one of the minor impurities, then the activity would be markedly higher than 2×10^5.

SUMMARY

1. Both culture filtrates of *Penicillium notatum* and extracts of mycelia, as well as the highly purified notatin (glucose oxidase) isolated from the former, in addition to their property of catalysing the oxidation of glucose, also have the property of accelerating its mutarotation.

2. The acceleration of mutarotation of glucose by these materials is due to the presence of a thermolabile and non-dialysable catalyst for which the name *mutarotase* is proposed.

3. That mutarotase is a catalyst distinct from glucose oxidase is supported by the following evidence. (*a*) They do not always coexist in the same highly purified notatin sample: while all samples (Y and Z) catalyse the oxidation of glucose only some of them (Z) promote mutarotation. (*b*) Sample Z, which contains both catalysts, can be fractionated to give preparations with a mutarotase/glucose oxidase ratio varying from zero to about three times that of Z. (*c*) Mutarotase shows a much greater stability at higher pH than glucose oxidase. (*d*) Mutarotase, unlike glucose oxidase, is strongly inhibited by excess of glucose and by carbohydrates structurally similar to glucose. (*e*) The mutarotase/oxidase ratio in some *Penicillium* culture filtrates is higher than that shown by notatin Z.

4. Under the experimental conditions adopted the catalytic coefficient of mutarotase (the calculated first-order mutarotation constant for glucose in a molar solution of the catalyst) is estimated to be at least 2×10^5 min.$^{-1}$ in presence of 4% glucose and would be greater at lower concentrations of glucose. This value is considerably higher than that for the OH^- ion ($8{\cdot}8 \times 10^3$ min.$^{-1}$) which is the most active catalyst of mutarotation hitherto known.

REFERENCES

Cecil, R. & Ogston, A. G. (1948). *Biochem. J.* **42**, 229.
Coulthard, C. E., Michaelis, R., Short, W. F., Sykes, G., Skrimshire, G. E. H., Standfast, A. F. B., Birkinshaw, J. H. & Raistrick, H. (1945). *Biochem. J.* **39**, 24.
Keilin, D. & Hartree, E. F. (1948*a*). *Biochem. J.* **42**, 221.
Keilin, D. & Hartree, E. F. (1948*b*). *Biochem. J.* **42**, 230.
Keilin, D. & Hartree, E. F. (1952). *Biochem. J.* **50**, 331.
Müller, A. (1931). *Ber. dtsch. chem. Ges.* **64**, 1820.
Smith, G. F. (1936). *J. chem. Soc.* p. 1824.
Smith, G. F. & Smith, M. C. (1937). *J. chem. Soc.* p. 1413.

Estimation of L-Malate and Fumarate by Malic Decarboxylase of *Lactobacillus arabinosus*

By P. M. NOSSAL* (1851 Overseas Scholar)
Department of Biochemistry, The University, Sheffield 10

(*Received* 5 *June* 1951)

The manometric estimation of L-malate by the malic decarboxylase of *Lactobacillus arabinosus* 17–5, has been described by Korkes & Ochoa (1948), Ochoa, Salles & Ortiz (1950) and Blanchard, Korkes, del Campillo & Ochoa (1950). As both the bacterial suspensions and the acetone powder extracts used by these workers have fumarase activity, the method measures the sum of malate and fumarate. In the present work, a modification is described whereby L-malate and fumarate are measured separately, and improvements are introduced which are based on the finding that in intact cells the activity of malic decarboxylase is optimal in the presence of glucose, manganous chloride and potassium chloride (Nossal, 1951*b*). Part of this work has been communicated to the Biochemical Society (Nossal, 1951*a*).

DEVELOPMENT OF THE METHOD

Standard conditions for the assay of malic decarboxylase activity, and estimation of L-malic acid in pure solution

The main compartment of conical Warburg vessels contained 3·6 ml. malate solution (equivalent to 40 μmol. DL-compound) and 0·1 ml. buffer-co-factor solution (1 g. glucose, 1 g. $MnCl_2.4H_2O$, 14·9 g. KCl and 16·5 ml. 3 M-sodium acetate buffer, pH 5·0, in 100 ml.). The side arm contained the bacterial suspension (0·2 ml.) prepared as previously described (Nossal, 1951*b*), and 0·1 ml. of buffer-co-factor solution. The gas space contained air. The bath temperature was 35°. These conditions had previously been found to be optimal for the quantitative decarboxylation of malate.

The rate of CO_2 evolution for 15 min. after mixing the contents of side arm and main compartment was proportional to the quantity of malic decarboxylase present, provided that the rate was not higher than 25 μl./min. The activity of the bacteria is expressed as μl. CO_2/mg. dry wt./hr. (Q_{CO_2}), calculated from the maximum rate of CO_2 evolution (generally attained in the period 5–15 min. after mixing). For the estimation of L-malic acid the same conditions are used, up to 3·5 ml. of the sample to be analysed (brought to pH 5) being placed in the main compartment.

The effect of growth conditions on the yield of malic decarboxylase. The basal medium used in the following experiments was that described previously (Nossal, 1951*b*). The yield of enzyme increased with the malate concentration of the medium up to a level of 0·1 M-DL-malate, above which growth was inhibited. The Q_{CO_2} values of cells obtained from media containing 4×10^{-3}, 2×10^{-2} and 0·1 M-DL-malate respectively were 205, 340 and 380, and the yields of cells (in mg. dry wt./100 ml. medium) were 61, 76 and 102 respectively.

The amount of enzyme produced after varying periods of incubation is shown in Table 1. It will be seen that production of the enzyme ceased earlier than growth. As the enzyme is an adaptive one, it was thought that the cessation of enzyme formation may have been due to the complete decomposition of the substrate. In fact, a second addition of 0·1 vol. 1 M-DL-malate after 12 hr. incubation increased the yield of enzyme at 24 hr. by about 30%. Incubation beyond 24 hr. did not increase the yield further. At 40° the yields of cells and enzyme were 10–20% lower than at 30°. Omission from the medium of acetate (which acts as a buffer) decreased the yield of enzyme by about 15%. In the standard procedure, therefore, the organism was grown at 30° for 24 hr. on the medium described previously, and after 12 hr. a second addition of 0·1 vol. sterile 1 M-DL-malate was made. The cells were washed with two lots of water (0·2 and 0·1 vol. of the medium respectively) and made up with 0·1 M-KCl to approximately 20% (wet wt.) suspensions.

Table 1. *Effect of growth period on yield of enzyme*

(1 ml. 20 hr. sub-culture inoculated into 500 ml. of medium (Nossal, 1951*b*). 100 ml. lots withdrawn at intervals, spun, washed twice, made up to 5·0 ml. with distilled water and stored at 2°. Activity of all samples estimated under the standard conditions after harvesting the last sample. Growth temperature 40°. Q_{CO_2} = μl. CO_2 evolved/mg. dry wt. cells/hr.)

Time of withdrawal (hr.)	Yield of cells/100 ml. (mg. dry wt.)	Q_{CO_2}	Enzyme yield per 100 ml. medium (mg. dry wt. $\times$ Q_{CO_2})
10	5·7	480	2720
14	13·0	372	4830
18	14·8	320	4740
22	16·5	293	4840
34	16·0	296	4740

The effect of enzyme concentration on the time required for complete decarboxylation. Under the standard conditions, 20 μmol. L-malate were quantitatively decarboxylated in 50–90 min. by 2·5 mg. (dry wt.) fresh cells, in 20–30 min. by 7 mg. and in 15 min. by 14 mg. Routinely, 4 mg. were used, so that 500 ml. medium yielded enough cells for 150 determinations. The time required for the complete decarboxylation of 20 μmol. L-malate by 4 mg. cells in the absence of inhibitory substances was about 45 min.

Specificity. Under the conditions described for the assay of decarboxylase activity (except that malate was replaced by other substances), 20 μmol. of the following compounds

* Present address: Department of Biochemistry, University of Adelaide, South Australia.

did not yield measurable amounts of CO_2 in the time required for the complete decarboxylation of 40 μmol. DL-malate: *dextro-iso*citrate, citrate, DL-lactate, L-α-hydroxyglutarate, α-hydroxy*iso*butyrate, DL-β-hydroxybutyrate, DL-citramalate ($CO_2H.C(OH)(CH_3).CH_2.CO_2H$), hydroxymalonate, DL-tartrate, α-keto-glutarate, succinate, maleate, malonate, thiomalate, disaccharides, hexoses and pentoses.

The only substances which formed CO_2 were fumarate, oxaloacetate and pyruvate. Fumarate reacted twenty to fifty times more slowly than equivalent amounts of malate, owing to the presence of a weak fumarase in the bacteria. The rate of decarboxylation of oxaloacetate was of about the same order as that of malate. The rate of CO_2 evolution from pyruvate differed greatly with pyruvate concentration.

Table 2. *Loss of activity of cell suspensions on storage at 2°*

(Standard conditions for assay of decarboxylase activity; 2 mg. dry wt. of cells used throughout; in all cases the stored cells were pre-incubated with the buffer-co-factor solution for about 1 hr. before mixing with the malate. The percentage loss of activity was calculated by comparing the rates of the CO_2 evolution for the first 30 min. after mixing with that originally observed with fresh suspensions.)

Exp. no.	Suspension medium for storage	Percentage loss of activity: 1st week	3rd week	5th week	7th week	9th week
1	Water	28	40	59	62	73
	0·5 M-KCl	11	34	35	32	43
	0·3 M-KCl	5	26	25	28	32
	0·1 M-KCl	0	18	20	20	25
2	Water	.	34	57	.	65
	0·1 M-KCl	.	0	8	.	15
	0·1 M-NaCl	.	12	18	.	20

Oxaloacetate can be removed by addition of 0·1 ml. of 0·4% aniline, which does not inhibit the decarboxylation of malate. Interference by fumarate and pyruvate is dealt with below.

Storage of the bacterial suspensions. Washed aqueous suspensions lose their malic decarboxylase activity fairly rapidly when stored at 2°; addition of glucose accelerates the inactivation, but KCl or NaCl greatly retard the loss of activity (Table 2). The activity can also be preserved in the deep freeze at −12°; freezing and thawing five times had little effect on the activity, especially when the cells were suspended in 0·1 M-KCl. Routinely, cells suspended in 0·1 M-KCl were stored at 2°, for up to 6 weeks.

Reactivation of suspensions. Samples of bacterial suspensions which had lost part of their activity owing to prolonged storage in water could in general be partially reactivated by the addition of cozymase (Table 3), or by pre-incubation at 30° in $2{\cdot}5 \times 10^{-2}$ M-acetate buffer, pH 5·0, with $2{\cdot}5 \times 10^{-3}$ M-glucose and 10^{-4} M-nicotinic acid for 60 min. Both findings indicate that some of the loss of activity can be attributed to loss of cozymase, but even large excesses of cozymase never restored the original activity completely. Thus other factors are also responsible for the inactivation.

Inhibition. The following substances (brought to pH 5 with HCl or NaOH if necessary), at the final concentrations indicated did not affect the decarboxylation appreciably: cyanide (10^{-2} M), fluoride (10^{-2} M), azide (10^{-2} M), sulphide (10^{-2} M), NaCl (M), NH_4Cl (M), malonate (10^{-2} M), maleate (10^{-2} M), succinate (10^{-2} M), fluoroacetate (10^{-2} M), hydroxylamine (10^{-2} M), semicarbazide (10^{-2} M), 8-hydroxyquinoline (10^{-3} M), *p*-nitrophenol (10^{-2} M), trichlorophenol (10^{-3} M), $BaCl_2$ (10^{-2} M), $ZnCl_2$ (10^{-2} M), octyl alcohol (saturated) and 'Tween 20' (0·2%).

Tungstate, trichloroacetate, trioctylamine, 2:4-dinitrophenol, iodoacetate, *n*-hexadecyltrimethylammonium bromide (cetavlon), $CuSO_4$, $Al_2(SO_4)_3$ and $FeSO_4$ were inhibitory at higher concentrations (Table 4). At lower inhibitor concentrations the inhibition was overcome by using greater amounts of cells except in the cases of cetavlon and trioctylamine.

Estimation of malate in the presence of fumarate. When equal quantities of fumarate and L-malate are present, e.g. 20 μmol. (equivalent to 445 μl. CO_2) of each, 10–20 μl. CO_2 are formed from fumarate over the period required for the complete decarboxylation of malate. A correction may be made for the presence of fumarate by measuring the CO_2 output over twice the period required for the complete decarboxylation of malate, which gives recoveries to within 5% of the theoretical (Table 5).

Most of the fumarase activity of washed cells suspended in 0·1 M-KCl disappeared on storage for 2–4 weeks at 2°, whilst the malic decarboxylase activity was largely retained. With such aged suspensions, the following recoveries from 40 μmol. DL-malate (equivalent to 445 μl. CO_2) were obtained in the presence of 20 μmol. fumarate, without applying correction factors (μl. CO_2): 442, 450, 446, 447, 443, 436, 452, 440, 445; mean: 444.

Table 3. *Reactivation of cell suspensions with cozymase*

(Standard conditions for assay of decarboxylase activity, but all cell samples pre-incubated in main compartment for about 1 hr. before mixing with malate.)

Exp. no.	Description of sample	Cells used (mg. dry wt.)	Period over which gas evolution was measured (min.)	Cozymase added* (mg.)	CO_2 evolution (μl.)
1	7 weeks old	2	30	.	219
		2	30	0·3	330
2	18 weeks old	4	90	.	232
		4	90	0·5	452 (complete)
3	15 weeks old	2	90	.	52
		2	90	0·3	243

* The sample used was prepared from yeast and was 50% pure.

Table 4. *Inhibition of the malic decarboxylase*

(Standard conditions for the estimation of malate; 40 μmol. DL-malate, inhibitors brought to pH 5 if necessary.)

Inhibitor	Final concentration (M)	Time for complete decarboxylation of uninhibited control (min.)	Time for complete decarboxylation in presence of inhibitor (min.)	Notes
Sodium tungstate	2×10^{-2}	40	.	Half-decomposition attained in 60 min.
	$2{\cdot}5 \times 10^{-3}$	40	50	.
Trichloroacetate	10^{-2}	40	90	.
	10^{-3}	40	40	.
Trioctylamine	1 drop/cup	45	.	Only 100 μl. evolved in 45 min.
2:4-Dinitrophenol	10^{-2}	40	.	Almost total inhibition
	10^{-3}	40	120	.
	10^{-4}	40	60	.
Iodoacetate	2×10^{-2}	40	100	.
	$2{\cdot}5 \times 10^{-3}$	40	70	.
n-Hexadecyltrimethyl-ammonium bromide (cetavlon)	10^{-2}	45	.	Almost total inhibition
	10^{-4}	30	.	Almost total inhibition
	10^{-4}	30	.	Almost total inhibition
$CuSO_4$	10^{-2}	30	80	.
	10^{-3}	30	30	.
$Al_2(SO_4)_3$	10^{-2}	30	.	Incomplete after 60 min.
	10^{-3}	30	50	.
$FeSO_4$	10^{-2}	30	60	.
	10^{-3}	30	40	.

An alternative procedure which eliminates the interference by fumarate is removal by chemical reactions. In pure solutions, excess saturated bromine water completely removed fumarate in 1 hr. at room temperature; this treatment, however, is unsatisfactory in biological material where it produces substances inhibitory to the malic decarboxylase. More generally applicable is the reduction of fumarate to succinate by Zn and H_3PO_4 (Krebs, Smyth & Evans, 1940). To 3·0 ml. of the sample, in a 10 ml. centrifuge tube, were added 0·1 g. granulated Zn (20–30 mesh), 0·10 ml. 0·1 M-$CuSO_4$, and 0·10 ml. 50% (w/v) H_3PO_4. After 45 min. at room temperature another 0·1 ml. portion of H_3PO_4 was added; reduction was complete within 90 min. and the sample was then brought to pH 5 by the addition of approx. 0·2 ml. 10 N-NaOH, using 0·1 ml. 0·04% bromocresol green as an internal indicator; water was added to give a final volume of 4·0 ml. After centrifugation, 3·0 ml. of the supernatant were used for the analysis. By this method, recoveries of malate from samples containing equal amounts of malate and fumarate were within 1–2% of the theoretical. The procedure proved satisfactory in biological materials.

Table 5. *Recovery of malate in the presence of fumarate*

(Standard conditions for the estimation of malate; 40 μmol. DL-malate; time required for complete decarboxylation of control: 40 min.)

Fumarate added (μmol.)	CO_2 evolution (μl.) 1st 40 min.	2nd 40 min.	Corrected value for malate	Percentage error
.	450	.	450	1
2·5	453	−1	454	2
5·0	469	8	461	3
10·0	474	11	463	4
20·0	486	25	461	3
40·0	513	50	463	4
80·0	548	89	459	3

Estimation of malate in the presence of pyruvate. If the sample to be assayed contains not more than 10 μmol. pyruvate, the additional CO_2 output due to pyruvate is small and steady, and a similar correction to that applied above for fumarate may be used, again giving recoveries within 5%. In an experiment with 20 μmol. DL-malate (equivalent to 223 μl.) and 10 μmol. pyruvate, the CO_2 evolution over the first 45 min. (during which complete decarboxylation was observed in a control without pyruvate) was 255 μl. and in the next 45 min. 39 μl. The malate recovery was thus 216 μl. (97%). In a second experiment with 40 μmol. DL-malate and up to 20 μmol. pyruvate the recoveries were 451–459 μl. (102–103%).

This correction cannot be applied to samples containing higher concentrations of pyruvate or small amounts of malate. In such cases, interference by pyruvate can be eliminated by addition of excess hydroxylamine or semicarbazide (Blanchard *et al.* 1950), for example, 10^{-2} M final concentration, neither of which appreciably affects the decarboxylation of malate. Recoveries to within 1% of the theoretical were obtained from samples containing 40 μmol. DL-malate and 30 μmol. pyruvate, by adding 35 μmol. of either hydroxylamine or semicarbazide. In the standard procedure 0·2 ml. 0·1 M-semicarbazide should be added to samples suspected to contain pyruvate.

Estimation of fumaric acid

When fumarase and fumarate are added to the malic decarboxylase system, fumarate is converted quantitatively to lactate and CO_2, the reaction proceeding to completion due to the continuous decarboxylation of the malate formed from fumarate. The fumarate content of a sample containing both malate and fumarate is given by the difference between an estimation done with added fumarase (which gives the sum of malate and fumarate) and a malate estimation on an aliquot in which fumarate has been removed by reduction or a malate estimation using stored fumarase-free cells.

Crude fumarase (from sheep heart) was rapidly inactivated by the decarboxylase system at pH 5·0 (see below), and a powerful fumarase preparation is therefore required. The following three yeast preparations proved suitable sources of fumarase: 1·0 ml. yeast maceration juice, as described by Krebs & Johnson (1937); 0·2–0·5 ml. of a 1:1 wet wt. aqueous suspension of baker's yeast frozen with solid CO_2 once and thawed at room temperature; 0·2 ml. of 1:1 wet wt. aqueous suspension of baker's yeast crushed in the cold without abrasives (Hughes, 1951). In all cases 0·2 ml. 0·2 M-NaF should be added to reduce the blank CO_2 production of these preparations which were (under the conditions of the estimation): 30–60 μl. for 1·0 ml. yeast maceration juice; up to 50 μl. for 0·5 ml. frozen-thawed baker's yeast suspension; up to 30 μl. for 0·2 ml. crushed baker's yeast suspension (all in the presence of fluoride). These blank values can be reduced to about 10 μl. by 3 hr. dialysis against running tap water, which causes but little loss of fumarase activity. None of the materials retain their fumarase activity for more than a few days at 2°, but they remain active for several months when stored at −12°.

For the estimation of small amounts of fumarate, the most suitable material was found to be the cloudy supernatant of a centrifuged suspension of crushed baker's yeast which had been dialysed for 3 hr. against running tap water at 20°; this material shows no blank gas output or uptake under the conditions of the estimation. Where the Hughes bacterial press is not available, dialysed suspensions of frozen-thawed baker's yeast are recommended.

Table 6 shows examples of estimations of malate and fumarate in separate solution; the cell suspension used had been stored in 0·1 M-KCl at 2° for 3 weeks and the crushed baker's yeast at −12° for 4 weeks. When using frozen-thawed baker's yeast suspension, an O_2 uptake was occasionally observed in the control cups, in which case the estimation was repeated anaerobically.

Inactivation of fumarases by Lb. arabinosus. Suspensions of *Lb. arabinosus* rapidly inactivate fumarases from both animal tissues and yeasts on incubation at pH 5·0. Table 7 shows an experiment in which yeast fumarase was incubated for 1 hr. at 30° with and without the bacterial suspension and its activity assayed after that time. It can be seen that 60% of the fumarase activity was lost by pre-incubation with the bacteria. No loss of malic decarboxylase activity was observed in control cups containing malate as the substrate. In another experiment, 90% inactivation was observed with 2 ml. hog-heart fumarase preparation (1:4 wet weight aqueous homogenate) after 2 hr. pre-incubation with the decarboxylase system. The mechanism of this inactivation has not been studied. For the estimation of fumarate by the decarboxylase method, a considerable excess of concentrated fumarase is added so that the estimation goes to completion before inactivation begins to interfere with it.

Recoveries of malate and fumarate from biological materials. Recovery was satisfactory in all cases, suggesting that there are not normally interfering substances present in biological material; examples of recovery experiments are shown in Table 8, from which it can be seen that with one exception the estimations were within ±2% of the theoretical.

Preparation of samples for analysis. Deproteinization is not necessary, but it may be required to destroy enzymes in samples to be analysed. This can be done by addition of 0·1 vol. 2 N-trichloroacetic acid and by placing this mixture in a boiling-water bath for 60 min. which destroys enzymes and at the same time removes trichloroacetic acid quantitatively. Care must be taken to note the change of volume of the sample during this treatment, and graduated test tubes were therefore used. Instead of trichloroacetic acid, 0·1 vol. 2 N-HCl may be added,

Table 6. *Course of* CO_2 *evolution during estimations of malate and fumarate*

(Standard conditions, using 5 mg. dry wt. cells, suspended in 0·1 M-KCl, and stored 3 weeks at 2°. The fumarase used was 0·2 ml. 1:1 wet wt. aqueous suspension of crushed baker's yeast, stored 4 weeks at −12°.)

Substrate (20 μM)	Fumarase added	Total CO_2 evolution (μl.) 10	20	30	40	50	60	70	80	90 min.
L-Malate	−	125	260	364	411	429	439	440	440	440
Fumarate	+	100	215	309	366	414	429	441	443	443
Fumarate	−	.	.	10	.	.	12	.	.	15

Table 7. *Inactivation of fumarase by pre-incubation with* Lactobacillus arabinosus

(Standard conditions for the estimation of malate plus fumarate; 0·2 ml. 1:1 crushed baker's yeast suspension in the main compartment of all cups. Cell suspension in one side arm and substrate in the other.)

Cup	Substrate	Comments	CO_2 evolution in first 60 min. (μl.)
1	DL-Malate (40 μmol.)	Cells tipped into main compartment after equilibration, substrate 1 hr. later	444 (complete)
2	Fumarate (20 μmol.)		163
3	DL-Malate (40 μmol.)	Cells and substrate tipped into main compartment 1 hr. after equilibration	442 (complete)
4	Fumarate (20 μmol.)		404

Table 8. *Recoveries of malate and fumarate from biological materials*

(The materials were prepared by grinding the tissue or plant with equal volumes of water and M-trichloroacetic acid. After centrifugation, trichloroacetic acid was removed by immersion of the supernatant for 60 min. in a boiling-water bath. Standard conditions for the estimation of malate and malate + fumarate.)

Material	Added substrate	Fumarase added	Total CO_2* evolved (μl.)
Sheep heart	40 μmol. DL-Malate	–	446
Sheep heart	20 μmol. Fumarate	+	440
Sheep heart	40 μmol. DL-Malate, 20 μmol. fumarate; reduced	–	444
Sheep heart	40 μmol. DL-Malate, 20 μmol. fumarate; reduced	+	450
Sheep heart	20 μmol. DL-Malate; 10 μmol. fumarate	+	452
Sheep heart	20 μmol. Fumarate; reduced; then 20 μmol. fumarate added	+	441
Guinea pig kidney	20 μmol. Fumarate	+	432
Tomato	20 μmol. Fumarate	+	455
Pear	40 μmol. DL-Malate	–	442
Swede	40 μmol. DL-Malate	–	446
		Mean	445

* Corrected for the malate and fumarate content of the biological material; the sheep heart was a washed mince and contained no measurable amounts of malate or fumarate.

followed by 2 min. immersion in a boiling-water bath. In both cases the pH of the sample is adjusted to about 5 with NaOH or HCl after heating.

APPLICATIONS OF THE METHOD

The malate and fumarate content of biological materials. Malate and fumarate were estimated in all materials in the supernatants of samples ground with sand and trichloroacetic or hydrochloric acids. The supernatants contained the same concentration of malate as the uncentrifuged whole mixtures. Animal tissues generally contained small amounts of the two acids, and only the sum of malate and fumarate was estimated. The following values (in μmol./g. wet weight) were obtained: guinea pig: muscle, 3–5; liver, 2–3; kidneys, 1–3; whole brain, 1. Pigeon: liver, 5–13; breast muscle, 1–4; heart, 1–2. Pigeon liver gave the highest values, a result probably connected with the presence of the highly active carbon dioxide-fixation enzyme systems. After tenfold concentration of extracts of sheep kidney and sheep liver by evaporation at 100° their malate and fumarate contents could be estimated: Sheep kidney contained 0·16 μmol. malate and 0·05 μmol. fumarate/g. wet weight and sheep liver 0·43 μmol. malate and 0·12 μmol. fumarate/g. wet weight. These give a ratio of malate to fumarate of about 3, which is approximately the expected value for tissues containing fumarase, and having a pH of 7·4. Marshall, Friedberg & Da Costa (1951) report malate concentrations of 0·36–3·43 mg./100 ml. (0·03–0·26 μmol./g.) in rat and mouse tissues.

Exceptionally high concentrations of malate were found in the haemolymph of *Gastrophilus intestinalis* larvae (collected during February), already known to contain relatively large concentrations of succinate (Levenbook, 1950). From 21 to 32 μmol. malate/ml. and from 3 to 6 μmol. fumarate/ml. were found. These acids, therefore, make up a large portion of the anion deficit of the haemolymph described by Levenbook. This author observed great variations in the concentrations of glucose, lactate and ammonia in the haemolymph, depending on whether the larvae were collected and washed at 38 or 0°; such variations were not observed for malate and fumarate.

All specimens of plant materials contained malate, but the amounts varied (μmol./g. wet wt.): apple, 200; rhubarb, 120; cabbage leaf, 95; shallot, 90; pear, 75; lemon, 70 (per ml. of juice); carrot, 55; onion, 50; celery stalks, 35; tomato, 30; orange, 25 (per ml. of juice); cauliflower leaf, 20; potato, 18; grapes, 17.

The diurnal variation of malic acid concentration in Crassulacean leaves (see Bennett-Clark, 1933; Krebs & Eggleston, 1944) was confirmed. The malate concentration of *Bryophyllum calycinum* leaves after exposure to daylight was 10 (in May), 23 (in February) μmol./g. wet weight, rose to 60–90 μmol./g. wet weight after keeping the plant in the dark for 16 hr., and returned to its original level on re-exposure to light. The leaves of other succulent plants also contained high concentrations of malate: *Sedum praealtum*, 20–28 μmol./g. wet weight; *Crassula argentae*, 50 μmol./g. wet weight.

Determinations of malate plus fumarate showed that none of the above fruits or leaves contained measurable amounts of fumarate (i.e. more than 5% of the malate value), but leaves of *Myrrhis odorata* and of *Glaucium flavum* contained 13 and 99 μmol. fumarate (and 15 and 46 μmol. malate/g. wet weight) respectively. Further experiments on the distribution of malate and fumarate in plants will be reported separately. Fumaric acid was found in *G. luteum* by Schmalfuss (1923) and in *Myrrhis odorata* by Itallie & Steenhauer (1926).

The detection of fumarase activity at pH 5·0

Materials were tested for fumarase activity at pH 5·0 as follows: up to 3 ml. of the material to be examined, brought to pH 5 with hydrochloric acid or sodium hydroxide, were placed in the main compartment of vessels containing, in addition, 0·1 ml. buffer-co-factor solution and 0·2 ml. 0·1 M-fumarate in the main compartment and 0·2 ml. cell suspension and 0·1 ml. buffer-co-factor solution in the side arm. Fumarase activity in the test system is shown by a steady carbon dioxide evolution on mixing (above the blank which contains no fumarate). In this way the presence of fumarase has been demonstrated in homogenates of mammalian and avian tissues. A suspension of ground *Gastrophilus intestinalis* larvae showed high fumarase activity; no fumarase activity was detected in extracts of leaves of *Bryophyllum calycinum*, *Sedum praealtum* and *Glaucium flavum*, in rhubarb petiole, *Helianthus tuberosus* tubers, or stored suspensions of intact cells of *Escherichia coli* and *Clostridium welchii*. These negative results do not prove the absence of fumarase from the materials examined, since pH 5 is well below the pH optimum of the enzyme.

The fumarase equilibrium. The method was used to measure the equilibrium ratio malate/fumarate at pH 5·0. At 30° a steady ratio was attained within an hour when 2·5 g. wet weight of frozen yeast was added to 200 μmol. fumarate. At 25°, 5 g. of yeast gave a constant ratio after 6 hr. A mean value of malate/fumarate at 25° of 10·1 (from seven experiments) was found by measuring separately malate and malate plus fumarate.

Results obtained at pH 7·4 agreed with the mean value of 3·54 reported earlier by Krebs *et al.* (1940). At 30°, equilibrium ratios (malate/fumarate) of 3·41 and 3·46 were obtained, again by separate measurement of malate only and malate plus fumarate.

DISCUSSION

The manometric method is more accurate, specific and sensitive than the polarimetric malate estimation (see Krebs & Eggleston, 1943, 1945). Amounts of L-malate from 1 μmol. upwards may be estimated, and the accuracy for amounts exceeding 5 μmol. is within $\pm 1\%$ with special precautions. In comparison with the fluorimetric method of Hummel (1948) and the subsequent modification of Marshall *et al.* (1951) which involves chromatographic separation of the malic acid, the manometric procedure is more specific, rapid and convenient. The malic decarboxylase method for measuring fumarate has advantages over the reduction-ether extraction technique of Krebs *et al.* (1940) in that it is more rapid and independent of the amount of succinate present. However, the decarboxylase method is inaccurate when the solution contains more than ten times as much malate as fumarate.

The main advantage of the method here described over Ochoa's procedure is the separate measurement of malate and fumarate. Both Blanchard *et al.* (1950) and Ochoa *et al.* (1950) report measurements of the sum of malate and fumarate only in mixed solutions. An additional advantage is the use of the cell suspensions made possible by the preserving action of potassium chloride. Suspensions and acetone powder extracts have similar specificities, and suspensions retain their activity well in 0·1 M-potassium chloride; therefore the preparation of acetone powder extracts, which involves a considerable loss of enzyme, is unnecessary; lastly, the use of bacterial suspensions makes unnecessary the addition of cozymase (required when acetone powder extracts are used for the estimation).

SUMMARY

1. The decarboxylase method for determining the sum of L-malate and fumarate (Ochoa *et al.* 1950; Blanchard *et al.* 1950) has been modified so that L-malate and fumarate can be measured separately.

2. Malic acid is measured manometrically using washed suspensions of malate-adapted cells of *Lactobacillus arabinosus* 17–5 at 35° in a solution of pH 5·0 containing $2{\cdot}5 \times 10^{-3}$ M-glucose, $2{\cdot}5 \times 10^{-3}$ M-manganous chloride and 10^{-1} M-potassium chloride.

3. Maximum yields of the enzyme are obtained by growing the organism for 24 hr. at 30° on media containing 10^{-1} M-DL-malate, with a second addition of 0·1 vol. M-DL-malate 12 hr. after inoculation.

4. Oxaloacetate, pyruvate and fumarate also form carbon dioxide. Oxaloacetate may be removed with aniline, pyruvate fixed with hydroxylamine or semicarbazide and fumarate removed by reduction with zinc and phosphoric acid, so that malate may be measured accurately in samples containing these acids.

5. Washed cells retain most of their malic decarboxylase activity for several weeks at 2° when suspended in 0·1 M-potassium chloride. Inactive cells may be partly reactivated by addition of cozymase or incubation with glucose and nicotinic acid.

6. Of some forty substances tested only *n*-hexadecyltrimethylammonium bromide (cetavlon), iodoacetate, tungstate, trichloroacetate, trioctylamine, 2:4-dinitrophenol, copper sulphate, aluminium sulphate and ferrous sulphate inhibit sufficiently at 10^{-2} M to interfere with the estimation; at 10^{-4} M only cetavlon inhibits severely.

7. The sum of fumarate and malate is measured by adding an excess of fumarase, and the fumarate content is obtained by difference.

8. Fumarase is progressively inactivated by suspensions of *Lactobacillus arabinosus* at pH 5·0 and 30°.

9. Animal tissues were found to contain between 1 and 13 μmol./g. wet weight malate plus fumarate. All plant materials tested contained malate (between 10 and 200 μmol./g. wet weight), whilst fumarate was absent from most materials. The occurrence of fumarate in *Myrrhis odorata* and *Glaucium flavum* (13 and 99 μmol./g. wet weight respectively) has been confirmed.

10. The haemolymph of *Gastrophilus intestinalis* larvae contained exceptionally high concentrations of malate and fumarate (24–38 μmol./ml.); these acids account largely for the anion deficit reported by Levenbook (1950).

The author wishes to express his gratitude to Prof. H. A. Krebs, F.R.S., for his constant criticism and advice, to Mr D. E. Hughes for helpful suggestions and for the cozymase used, and to Miss E. Ellis for assistance during the preliminary experiments.

REFERENCES

Bennett-Clark, T. A. (1933). *New Physiol.* **32**, 39, 128, 197.
Blanchard, M. L., Korkes, S., del Campillo, A. & Ochoa, S. (1950). *J. biol. Chem.* **187**, 875.
Hughes, D. E. (1951). *Brit. J. exp. Path.* (in the Press).
Hummel, J. P. (1948). *J. biol. Chem.* **180**, 1225.
Itallie, L. van & Steenhauer, A. J. (1926). *Chem. Zbl.* **1**, 1408.
Korkes, S. & Ochoa, S. (1948). *J. biol. Chem.* **176**, 463.
Krebs, H. A. & Eggleston, L. V. (1943). *Biochem. J.* **37**, 334.
Krebs, H. A. & Eggleston, L. V. (1944). *Biochem. J.* **38**, 426.
Krebs, H. A. & Eggleston, L. V. (1945). *Biochem. J.* **39**, 408.
Krebs, H. A. & Johnson, W. A. (1937). *Biochem. J.* **31**, 645.
Krebs, H. A., Smyth, D. H. & Evans, E. A. jun. (1940). *Biochem. J.* **34**, 1041.
Levenbook, L. (1950). *Biochem. J.* **47**, 336.
Marshall, L. M., Friedberg, F. D. & Da Costa, W. A. (1951). *J. biol. Chem.* **188**, 97.
Nossal, P. M. (1951*a*). *Biochem. J.* **48**, xviii.
Nossal, P. M. (1951*b*). *Biochem. J.* **49**, 407.
Ochoa, S., Salles, J. B. V. & Ortiz, P. J. (1950). *J. biol. Chem.* **187**, 863.
Schmalfuss, H. (1923). *Hoppe-Seyl. Z.* **131**, 166.

The Composition of the Bulk Proteins of *Chlorella*

By L. FOWDEN
Department of Botany, University College, Gower Street, London, W.C. 1

(*Received* 20 *June* 1951)

Little information is at present available regarding the nature of the algal proteins. Mazur & Clark (1938, 1942) have given the amino-acid composition of the proteins of several species of marine algae, and one fresh-water alga. However, Lugg (1949) has recently questioned the accuracy of the values obtained, and doubted the possible phylogenetic implications. Hot formic acid was used to extract the proteins from the defatted algae, and since this procedure was only 70–80 % efficient, the resulting protein fractions may not have been representative of the whole. Analyses of the acid hydrolysates of the extracted material accounted for 76–88 % of the total protein nitrogen, though of this nitrogen as much as 20 % was combined in the form of humin. No other complete analyses of algal proteins appear to have been published.

This paper presents the results of an investigation of the amino-acid composition of the bulk protein of the unicellular alga, *Chlorella vulgaris*, and is a continuation of the qualitative work reported earlier (Fowden, 1951*b*). The analyses were made on a protein fraction believed to be representative of the whole, using the technique of paper chromatography.

EXPERIMENTAL

Material. Chlorella vulgaris was grown on the medium of Pearsall & Loose (1936) in penicillin culture flasks maintained at 25° under continuous illumination; the flasks were occasionally gently shaken. When nearing the end of its phase of exponential growth (6–7 days), the *Chlorella* was harvested by centrifugation at 500 *g*, and then twice washed with distilled water.

Protein extraction. The method used was based on the borate-ether-ethanol method of Lugg (1939). The freshly harvested *Chlorella* cells (about 2 g. on a dry-wt. basis) were suspended in 35 ml. of sodium borate solution (1·1 g./l.), and then the cell walls were broken, releasing the cytoplasmic proteins, by applying a shearing stress of 20,000 lb./sq.in. (Milner, Lawrence & French, 1950). Haemocytometer counts showed that 8–10 % of the cells remained unbroken after this treatment. A sample of the suspension was taken for total-nitrogen assay. The bulk of the suspension was diluted to 250 ml. with more sodium borate solution and 250 ml. of an ether-ethanol mixture (1 vol. ether to 4 vol.

ethanol) at about 5° were then slowly added with good stirring. This treatment disrupted the chloroplasts, and their protein passed into solution. After standing for 1 hr., the mixture was centrifuged for 30 min. at 500 *g*. The supernatant was poured off and the separated solid material (fraction *A*) was washed, dried and stored for nitrogen assay. The supernatant and washings were then adjusted to pH 4·5 with acetic acid, and warmed to 70° to flocculate the protein. The protein was separated after cooling by centrifugation, and the supernatant (fraction *B*) was set aside for nitrogen assay. The protein was washed in turn with dilute acetic acid (pH 4·5), boiling ethanol (twice), dilute citric acid, boiling ethanol (twice), and finally ether. It was then air dried, weighed and finely ground to a light buff-coloured powder (fraction *C*). Nitrogen determinations were made on a sample of the protein, and upon the combined washings (fraction *D*).

Protein hydrolysis. The protein was hydrolysed for 24 hr. at 105° in a sealed ampoule using 5 ml. of a mixture of equal vol. of 10 N-HCl and glacial acetic acid, and following a suggestion of Kofranyi (1948), $SnCl_2.2H_2O$ (4% w/v) was added. The protein concentration during the hydrolysis was about 10 mg./ml. The addition of the $SnCl_2$ completely prevented the formation of insoluble humin, and the final hydrolysate had a pale-yellow colour. The mixture was evaporated to dryness *in vacuo* at laboratory temperature.

The residue was dissolved in about 5 ml. of distilled water, and then desalted by the method of Consden, Gordon & Martin (1947). The pH of the solution rapidly increased during the desalting, and the remaining soluble tin was precipitated as $Sn(OH)_2$. This was removed by centrifugation, and washed several times with small volumes of distilled water. The supernatant and washings were combined, and evaporated to dryness *in vacuo*. The residue was dissolved in 5 ml. water; 100 μl. of the solution were used for each chromatogram.

Rees (1946) has reported some loss of pure threonine and serine under the normal conditions of hydrolysis of proteins in 6 N-HCl, but under the hydrolysis conditions used here, there was no detectable loss of threonine, and a smaller (6·0%) loss of serine than that reported by Rees. The values given later for serine have been corrected for this loss.

Since some insoluble $Sn(OH)_2$ was produced during desalting, it seemed quite probable that some of the dicarboxylic amino-acids present in the hydrolysate may have been absorbed by this basic precipitate, resulting in estimates that were too low for these amino-acids. In order to check this point they were also determined after hydrolysing the protein in 6 N-HCl for 24 hr. at 105°. Though in this case 4% of the total protein nitrogen was lost as insoluble humin nitrogen, the values obtained for the aspartic and glutamic acid contents of the hydrolysate were accepted as true estimates of the amounts present in the protein, since these two amino-acids are thought to play no significant role in humin production. The aspartic and glutamic acid contents found by this procedure were approximately 50% higher than those determined after desalting in the presence of $SnCl_2$.

Stein & Moore (1951) have reported an appreciable conversion of arginine to ornithine during prolonged desalting of amino-acid-salt mixtures, but the conversion occurring under the conditions described above must have been less than 5%, as demonstrated by chromatographic analysis of arginine solutions desalted under the same conditions, and by an independent assay of arginine by the Sakaguchi method described by Brand & Kassell (1942). No losses of the other amino-acids present in the hydrolysate were encountered during desalting.

Nitrogen determinations. Total N determinations were obtained using the micro-Kjeldahl method of Chibnall, Rees & Williams (1943). Amide N values were obtained by estimating the NH_3 formed during hydrolysis with N-H_2SO_4 for 3 hr. at 100°. α-NH_2-N determinations were made by the colorimetric copper method of Woiwod (1949).

Amino-acid assays. These were performed as described by Fowden (1951*a*) after the complete separation of each amino-acid had been achieved on Whatman no. 4 paper chromatograms.

Chromatographic technique. This was essentially that of Consden, Gordon & Martin (1944). The separation of the amino-acids was achieved entirely on one-dimensional chromatograms using three solvent mixtures. Since a suitable source of ultraviolet light was not available, all spots were located and cut out by comparison with an identical parallel chromatogram (Martin & Mittelmann, 1948). The recovery of the amino-acids was checked at regular intervals by running known control mixtures on the same sheet of paper and, in the case of amino-acids such as methionine, where the recovery of these standards was constant but below the theoretical amount, the values given for the protein were based on these estimated recoveries.

Solvents. Aspartic acid, glutamic acid, serine, glycine and threonine were separated as well defined spots after running for 60 hr. in phenol saturated with 0·1% NH_3 (w/v) solution. Any cystine present in the hydrolysate was oxidized to cysteic acid by H_2O_2 (Dent, 1947) before the commencement of the run. The NH_3 concentration in the water phase placed in the bottom of the tank was increased to 0·6% (w/v) to give better separations of these amino-acids.

Alanine, tyrosine and valine were separated by running for 30 hr. in the *n*-butanol-acetic acid mixture of Partridge (1948). Methionine, which would otherwise tend to overlap valine, was oxidized by H_2O_2 to the slower moving sulphone.

Lysine, histidine and arginine were also separated using this *n*-butanol-acetic acid mixture. It was found advisable to use a mixture which had been equilibrated for 5–6 days before separating the two phases. The R_F values were then lower than in the freshly prepared solvent and the resolution of the three amino-acids was much better. Good separation was achieved at the end of a 5-day run.

Proline, methionine, phenylalanine, isoleucine and leucine were separated using water-saturated *tert.*-amyl alcohol (technical). 1% (v/v) diethylamine was added to the aqueous phase in the bottom of the tank and each chromatogram was developed for 5 days. The amino-acids are mentioned above in the order of their increasing R_F values for the respective solvents. It was noticed that phenylalanine did not occupy the same relative position in *tert.*-amyl alcohol as that described by Work (1949), but this was probably due to differences in the purity of the alcohol used. The dilute alkali treatment used to remove NH_3 from the paper before amino-acid assay (Fowden, 1951*a*), was found to remove efficiently all of the diethylamine absorbed on the paper. Proline was estimated after chromatographic separation by the method of Woiwod (1949), since the ninhydrin method of estimation is very insensitive for this amino-acid.

Cystine could not be estimated as cysteic acid on chromatograms without overloading the paper because of the relatively small amount of this amino-acid present in the protein. It was estimated in duplicate 1 ml. samples of the

hydrolysate by the method of Sullivan (1929). Tryptophan was estimated using *p*-dimethylaminobenzaldehyde (Ehrlich's reagent) after 5 N-NaOH hydrolysis of the protein for 30 hr. at 100°. A correction was applied for an estimated loss of 5% during the course of the hydrolysis (Lugg, 1938).

RESULTS

The sample of *Chlorella* used in this investigation contained 4·3% nitrogen on a dry-weight basis. Repeated extraction of the dried cells with 70% (v/v) aqueous ethanol at room temperature showed 10·3% of this to be soluble non-protein nitrogen.

Table 1 shows the distribution of total nitrogen between the fractions *A*, *B*, *C* and *D* obtained during the protein extraction, together with the probable nature of the fractions.

Table 1. *The distribution of total nitrogen between the fractions obtained during the protein extraction*

Fraction	Percentage total nitrogen	Composition of fraction
A	10·2	Broken cell wall material and 8–10% unbroken cells
B	12·4	Soluble nitrogenous compounds, carbohydrates, lipids, etc.
C	75·8	Protein with carbohydrate impurities
D	1·4	Chlorophyll eluted from protein, some carbohydrate, etc.

The nitrogen in fraction *A* was probably all present in the unbroken cells, from which no protein would be released. Of the nitrogen released from the broken cells (nitrogen appearing in fractions *B*–*D*), 85% appeared as protein nitrogen in fraction *C*. The extraction of the dried cells with 70% ethanol had indicated that 89·7% of the total nitrogen was present as protein nitrogen. Since good agreement was found between these two figures the extracted protein was considered to be representative of the proteins of the whole cells. The nitrogen content of this protein fraction was 11·5%.

Table 2 gives the results of the amino-acid analyses performed on hydrolysates of this protein fraction. The figures obtained using paper chromatography represent the means of six determinations. Cystine and tryptophan values are the means of duplicate estimations. The probable errors of the values quoted are given in parentheses. The table also includes for comparison the amino-acid composition of the bulk proteins obtained from leaves of the Gramineae family (from Lugg, 1949), and the composition of an acid hydrolysate of the nitrogen fraction of a brewer's yeast which was insoluble in 75% ethanol (Lindan & Work, 1951).

The arginine-nitrogen figure obtained by the Sakaguchi method was 15·5%. The figures in Table 2 show that 73·9% of the total protein nitrogen is present as α-amino nitrogen after hydrolysis. A direct estimate of the α-amino nitrogen in the desalted acid hydrolysate made by the method of Woiwod (1949) gave a value of 74·4% after correction for the losses of aspartic and glutamic acids occurring during the desalting.

Table 2. *The amino-acid composition of the bulk protein of* Chlorella vulgaris *compared with similar data for brewer's yeast and higher plants*

(Figures in brackets indicate probable error.)

	g. amino-acid N/100 g. protein N		
Amino-acid	*Chlorella vulgaris*	Brewer's yeast (Lindan & Work, 1951)	Gramineae (Lugg, 1949)
Aspartic acid	6·4 (0·3)	8	4·9–5·4
Glutamic acid	7·8 (0·3)	10	6·6–7·8
Serine	3·3 (0·2)	4	—
Threonine	2·9 (0·15)	7	3·0
Glycine	6·2 (0·3)	8	0·4
Alanine	7·7 (0·3)	10	4·4–5·1
Valine	5·5 (0·2)	8	3·3–4·2
Leucine	6·1 (0·3)	6	7·1–8·8
Isoleucine	3·5 (0·2)	4	
Phenylalanine	2·8 (0·1)	1·6	2·5–2·6
Tyrosine	2·8 (0·1)	2	2·3–2·5
Proline	7·2 (0·3)	3	3·1
Tryptophan	2·1	0	1·8–2·1
Methionine	1·4 (0·1)	0·5	1·4–1·6
Cystine	0·2	0·9	1·3–1·5
Arginine	15·8 (0·8)	10	13·7–14·3
Histidine	3·3 (0·15)	8	3·6–3·7
Lysine	10·2 (0·35)	9	6·3–6·6
Amide N	6·1	—	4·7–5·3
Total N	101·3	100	74·4 (mean)

DISCUSSION

The results presented in Table 2 give a very complete analysis of the protein of *Chlorella*, the probable errors of the values obtained by the paper-chromatographic technique being about 5% of the individual mean values. The protein analysed was extracted from rapidly growing cells, and is marked by its high content of basic amino-acids. In contrast to the findings of Mazur & Clark (1938) for other algae, all the usual amino-acids were found to be present.

When the composition of the *Chlorella* protein is compared with that of the proteins in the leaves of higher plants, it is seen that a close similarity exists between the values for many amino-acids, the major differences occurring in the values given for the monoamino-monocarboxylic acids and proline. Since the analyses of the leaf proteins are only 75% complete, it seems possible that the values given by Lugg (1949) for these amino-acids were underestimated by the rather unspecific methods

available prior to partition chromatography. If this were the case, the only outstanding difference between the composition of leaf proteins and the algal protein reported here is in the very low cystine content of the latter. The composition of the yeast protein also shows many similarities with that of *Chlorella*, the values for the monoamino-monocarboxylic acids again being much higher than those reported for the leaf proteins.

SUMMARY

1. A representative sample of the proteins of the alga *Chlorella vulgaris* was prepared.

2. The amino-acid composition of this protein fraction was investigated by the technique of paper-partition chromatography after an acid hydrolysis during which no humin formation occurred.

3. The analysis accounted for 101·3% of the total protein nitrogen.

4. The amino-acid composition of this algal protein fraction is compared with figures available for the composition of the proteins obtained from the leaves of the higher plants and of the proteins found in a sample of a brewer's yeast.

REFERENCES

Brand, E. & Kassell, B. (1942). *J. biol. Chem.* **145**, 359.
Chibnall, A. C., Rees, M. W. & Williams, E. F. (1943). *Biochem. J.* **37**, 354.
Consden, R., Gordon, A. H. & Martin, A. J. P. (1944). *Biochem. J.* **38**, 224.
Consden, R., Gordon, A. H. & Martin, A. J. P. (1947). *Biochem. J.* **41**, 590.
Dent, C. E. (1947). *Biochem. J.* **41**, 240.
Fowden, L. (1951*a*). *Biochem. J.* **48**, 327.
Fowden, L. (1951*b*). *Nature, Lond.*, **167**, 1030.
Kofranyi, E. (1948). *Hoppe-Seyl. Z.* **283**, 14.
Lindan, O. & Work, E. (1951). *Biochem. J.* **48**, 337.
Lugg, J. W. H. (1938). *Biochem. J.* **32**, 775.
Lugg, J. W. H. (1939). *Biochem. J.* **33**, 110.
Lugg, J. W. H. (1949). *Advanc. Prot. Chem.* **5**, 229.
Martin, A. J. P. & Mittelmann, R. (1948). *Biochem. J.* **43**, 353.
Mazur, A. & Clark, H. T. (1938). *J. biol. Chem.* **123**, 729.
Mazur, A. & Clark, H. T. (1942). *J. biol. Chem.* **143**, 39.
Milner, H. W., Lawrence, N. S. & French, C. S. (1950). *Science*, **111**, 663.
Partridge, S. M. (1948). *Biochem. J.* **42**, 238.
Pearsall, W. H. & Loose, L. (1936). *Proc. roy. Soc.* B, **121**, 451.
Rees, M. W. (1946). *Biochem. J.* **40**, 632.
Stein, W. H. & Moore, S. (1951). *J. biol. Chem.* **190**, 103.
Sullivan, M. X. (1929). *Publ. Hlth Rep., Wash.*, suppl. 78.
Woiwod, A. J. (1949). *Biochem. J.* **45**, 412.
Work, E. (1949). *Biochim. Biophys. Acta*, **3**, 400.

The Branched-chain Fatty Acids of Butterfat

2. THE ISOLATION OF A MULTI-BRANCHED C_{20} SATURATED FATTY ACID FRACTION

By R. P. HANSEN and F. B. SHORLAND
Fats Research Laboratory, Department of Scientific and Industrial Research, Wellington, New Zealand

(*Received* 18 *July* 1951)

In Part 1 of this series (Hansen & Shorland, 1951) the occurrence of small amounts of branched-chain acids in butterfat was indicated and the isolation of two C_{17}-methyl branched-chain acids, isomeric with normal heptadecanoic acid, was reported.

Further investigation of the acetone-soluble components separated at low temperature from hydrogenated methyl esters of the C_{18} acids of butterfat, has revealed the presence of a multi-branched C_{20} acid fraction.

EXPERIMENTAL

As reported earlier (Hansen & Shorland, 1951) a concentrate (4·4 kg.) of methyl esters of C_{18} acids prepared from butterfat was crystallized from acetone at −30°. The soluble portion after hydrogenation was again crystallized from acetone at −30° yielding 87·6 g. of acetone-soluble material with iodine value 8·3. This latter fraction was distilled *in vacuo* in an 8 ft. Vigreux column yielding eight fractions, EL1 to EL8, together with a viscous residue ELR (compare Table 1, Hansen & Shorland, 1951). From these eight fractions were derived the C_{17} branched-chain acids previously reported, together with all subsequent fractions referred to in this paper. By repetition of the processes of selective bulking of those fractions with similar molecular weight, followed by fractionation, a series of fractions was obtained which could not be further resolved by the columns at our disposal and which were combined to constitute E4 (9·6 g. methyl esters, mean saponification equivalent 303·4).

The methyl esters of E4 were crystallized from 20 vol. of methanol at −37° yielding: (*a*) E4L 5·56 g., soluble

Table 1. *Fractionation of methyl esters* (E7L, 1·5 *g.*)

Fraction	Weight (g.)	B.p. (°) (approx. 0·1 mm.)	Saponification equivalent	Refractive index	Iodine value	
E7L1	0·06	120–125	—	—	—	—
E7L2	0·30	125–128	328·2	$n_D^{20°}$ 1·4425	—	Liquid
E7L3	0·95	128	329·9	$n_D^{20°}$ 1·4426	1·6	Liquid at −27°
E7L4	0·19	128	330·5	$n_D^{20°}$ 1·4431	—	Liquid

Table 2. *Chemical and physical properties of fatty acid fraction* E9

Fraction	Wt. (g.)	Sap. equiv.	Iodine* value (Wijs)	C-methyl†	Combustion analysis	M.p. (°)	Refractive index	Optical rotation
E9 (acid)	1·05	312·7	0·4	13·09%	C 77·4% H 12·9%	Below −70	$n_D^{20°}$ 1·4500	Nil

* $E^{1\%}_{1\,cm.}$ 234 mμ. value was 5·9, indicating the presence of only trace amounts of conjugated unsaturation.
† Analyses by Weiler & Strauss, Oxford, England.

Table 3. *Chemical and physical properties of fatty acid fraction* Eq1bL

Fraction	Wt. (g.)	Sap. equiv.	Iodine value	C-Methyl*	Combustion analysis*	Refractive index	M.p. (°)	Optical rotation
Eq1bL (acid)	0·19	314·5	Nil	13·4%	C 77·2% H 13·1%	$n_D^{20°}$ 1·4513	Below −70	Nil

* Analyses by Weiler & Strauss.

fraction, liquid at room temperature, saponification equivalent 310·5. (*b*) E4S 4·02 g., insoluble fraction, also liquid at room temperature, saponification equivalent 294·7.

Chromatographic adsorption on activated alumina of E4L dissolved in light petroleum (b.p. 50–60°), served to remove traces of impurities and effected separation into nineteen fractions. Two of these fractions E4Lb 0·89 g. $n_D^{20°}$ 1·4424, and E4Lc 1·28 g. $n_D^{20°}$ 1·4425, which had been eluted with light petroleum (b.p. 50–60°) were combined together and denoted E7 (saponification equivalent 324·3).

Crystallization from 20 vol. of methanol at −60° resolved E7 into two fractions: (*a*) E7L 1·88 g., soluble fraction, liquid at room temperature, m.p. below −35°, and (*b*) E7S 0·04 g., insoluble fraction, solid at room temperature.

E7L (saponification equivalent 325·1, iodine value (Wijs) 1·1) when dissolved in 20 vol. of light petroleum (b.p. 50–60°) gave no precipitate at −70°. Fractionation of 1·5 g. E7L methyl esters in a microcolumn yielded four fractions as shown in Table 1.

Fraction E7L3 was crystallized from 20 vol. acetone at −60° giving (*a*) E7L3L 0·77 g., soluble fraction, and (*b*) E7L3S 0·01 g., insoluble fraction, E7L3L was completely soluble in 20 vol. light petroleum at −70°.

E7L3L and E7L2 were bulked together as acids and denoted E9, the chemical and physical characteristics of which are recorded in Table 2.

A second fraction Eq1bL, possessing properties closely agreeing with those of E9 has been derived by similar but independent processes from the original fractions already described (compare Table 1, Hansen & Shorland, 1951). Characteristics of fraction Eq1bL are recorded in Table 3.

DISCUSSION

The two fractions isolated (E9 and Eq1bL) display almost identical chemical and physical properties. Analytical data are in agreement with the empirical formula $C_{20}H_{40}O_2$, the theoretical values being C, 76·9; H, 12·9%; saponification equivalent 312·5.

The recorded C-methyl values for E9 and Eq1bL are equivalent to 2·78 and 2·81 mol. of acetic acid, respectively. These figures when interpreted according to the evidence of Ginger (1944), indicate the presence of at least three and possibly four methyl or homologous groups.

The structure of the multi-branched C_{20} acid fraction reported in this paper has not been established. Its extraordinarily low melting point (below −70°) contrasts it with Velick's (1944) phytomonic acid, $C_{20}H_{40}O_2$, which has a single methyl side chain and a melting point of 24° (more than 50° lower than that of the corresponding straight-chain acid eicosanoic, melting point 75·3°). From the evidence presented by Cason & Winans (1950) on the methyl substituted isomers of octadecanoic acid, it may be inferred that a low melting point will indicate branching near the centre of the straight chain. Multiple branching can be expected to be accompanied by further lowering of the melting point.

Characteristic of the fraction isolated is its extreme solubility in different solvents, suggesting that both the number of side chains and their positions profoundly influence the solubility of saturated acids.

The very low solidifying point of this C_{20} saturated branched-chain acid has precluded melting-point determinations, and in the absence of these criteria of homogeneity, freedom from isomers or closely related substances cannot be assured. When larger quantities of the substance become available, however, derivatives will be prepared and further resolution attempted.

SUMMARY

A C_{20} saturated acid fraction containing at least three and possibly four methyl groups has been isolated from butterfat.

The authors wish to acknowledge technical assistance by Miss N. J. Cooke.

REFERENCES

Cason, J. & Winans, W. R. (1950). *J. org. Chem.* **15**, 139.

Ginger, L. G. (1944). *J. biol. Chem.* **156**, 453.

Hansen, R. P. & Shorland, F. B. (1951). *Biochem. J.* **50**, 207.

Velick, S. F. (1944). *J. biol. Chem.* **152**, 533.

The Oxidation of Amines by Extracts of Pea Seedlings

By R. H. KENTEN and P. J. G. MANN

Biochemistry Department, Rothamsted Experimental Station, Harpenden, Hertfordshire

(*Received* 2 *June* 1951)

While a number of enzymes catalysing reactions which lead *in vitro* to hydrogen peroxide formation have been shown to be present in animal tissues little is known about the distribution of such enzymes in higher plants. These enzymes may be of importance not only because of the oxidations they catalyse, but also because the hydrogen peroxide formed can be used by peroxidase (Thurlow, 1925) or catalase (Keilin & Hartree, 1936, 1945) for coupled oxidations. In this connexion Kenten & Mann (1949, 1950) showed that peroxidase systems can oxidize manganese and suggested that hydrogen peroxide produced by flavoprotein enzymes could be used *in vivo* for this oxidation. In the present work colorimetric evidence has been obtained suggesting the presence of enzyme systems producing hydrogen peroxide in extracts of many plants. In an investigation of such enzyme systems in extracts of pea seedlings it has been found that not only diamines (Werle & Pechmann, 1949), but also certain monoamines are oxidized by these extracts with formation of hydrogen peroxide. The oxidation of the monoamines is apparently not due to the plant monoamine oxidase described by Werle & Roewer (1950).

MATERIAL AND METHODS

Plant material. The majority of the plants examined were glasshouse grown in a John Innes potting compost. The rest of the plants were from gardens in the neighbourhood of Rothamsted. The peas and beans were soaked for 24 hr. in water prior to sowing. In some experiments with peas when seedlings only a few days old were required the peas were germinated in moist washed sand. Pea seedlings were also grown aseptically in boiling tubes on a mineral salts agar medium. Before sowing on this medium the seed coats were sterilized by treatment first with ethanol and then with 0·2% $HgCl_2$ washed off with several changes of sterile water (Thornton, 1930).

Enzyme preparations. Water extracts of the plant material and a dry preparation obtained by precipitation of such extracts with $(NH_4)_2SO_4$ and acetone were used.

(*a*) *Plant extracts.* The plant material was washed free from soil and ground in a mortar with sand and water, squeezed by hand through madapollam and finally made up to approximately double the wet weight.

(*b*) *Dry prepration.* Pea seedlings (6–18 days old) were washed free from soil and any obviously diseased material removed. With the older seedlings it was frequently necessary to remove a number of the cotyledons on this account. About 100 g. of seedlings were extracted as above; the extract was then fractionally precipitated with $(NH_4)_2SO_4$ and the precipitate obtained with 30–66% saturation was dialysed overnight against 2 l. of distilled water. The dialysed suspension was centrifuged and the supernatant cooled to 0° and treated with 2 vol. of acetone cooled to −10°. The precipitate was centrifuged off and dried *in vacuo*. The yield was 400–600 mg. The activity of the various fractions was estimated manometrically by measuring the increase in O_2 uptake produced by 0·01 M-β-phenylethylamine in 0·067 M-orthophosphate at pH 8·5. The supernatant obtained after dialysis of the $(NH_4)_2SO_4$ precipitate contained 60–70%, and the acetone precipitate 30–40%, of the activity of the original plant extract. The dry preparation was stored *in vacuo* at room temperature, and under these conditions about 30% of the activity towards amines was lost in a fortnight.

Manometric measurements were carried out in air in the Warburg apparatus at 25°. The volume of the reaction mixture was 3 ml. and KOH was present in the centre cup unless otherwise stated.

Catalase was prepared from horse liver by a method based on that of Agner (1938). The activity of the preparation was estimated by the method of Sumner & Somers (1943). The rate of decomposition of 0·01 N-H_2O_2 in 0·0067 M-orthophosphate at pH 7 at 0°, by a suitable amount of catalase, was followed by iodine titration of samples taken at 0, 3, 6, 9 and 12 min. From the equation

$$K = \frac{1}{t} \log_{10} \frac{a}{a-x},$$

where a is the initial H_2O_2 concentration and $a - x$ is the concentration at time t in minutes, K values for the time intervals were calculated and the K value at zero time obtained by extrapolation. Using this value for K the Katalasefähigkeit (Kat.f. $= K/g.$ enzyme) was found to be 6000.

Amines were used as the hydrochlorides.

Estimation of aldehydes was done with the bisulphite method of Clift & Cook (1932).

Estimation of ammonia. A preliminary distillation was carried out in the Markham (1942) still from borate buffer pH 9·5. The NH_3 in the distillate was absorbed on to yellow HgO according to the method of Pugh & Quastel (1937). The washed HgO-NH_3 complex was then transferred to the still and decomposed by the addition of 2·5 ml. of 5 N-NaOH. The distillate was collected in boric acid and titrated with N/140 HCl.

EXPERIMENTAL AND RESULTS

Colorimetric evidence of hydrogen peroxide production

The blue coloration obtained when benzidine and H_2O_2 are added to most plant and animal tissues is known to be due to the oxidation of benzidine by peroxidase or to the peroxidase-like action of haem or haematin derivatives. Therefore, a blue coloration obtained with tissue and benzidine in the absence of added H_2O_2 suggests the formation of H_2O_2 by the tissue. Goldacre (1949) found that extracts of pea epicotyls gave a blue coloration with benzidine in absence of added H_2O_2. This observation has been confirmed and positive tests have been obtained with pea roots and cotyledons as well as epicotyls, but under the conditions used the blue colour was frequently faint and faded rapidly. Owing to the catalatic activity of plant extracts the H_2O_2 formed is unlikely to accumulate. If the extracts were incubated with added Mn^{++} and pyrophosphate at pH 7 the intensity of the subsequent benzidine test was much increased. In pyrophosphate solution the H_2O_2 formed by the plant extracts can be used by peroxidase systems to oxidize added Mn^{++} to Mn^{+++}, which may accumulate as manganipyrophosphate. Since manganipyrophosphate oxidizes benzidine such an accumulation would explain the great increase in intensity of the benzidine test.

The colorimetric test was negative with extracts which had been heated for 5 min. in a boiling-water bath, and its intensity was considerably reduced by the previous addition of catalase to the extracts.

The tests were made in 25 ml. beakers with reaction mixtures consisting of 0·5–1 ml. plant extract, 0·5 ml. 0·2 M-pyrophosphate (pH 7), with and without 0·2 ml. 0·01 M-$MnSO_4$, and water to 3 ml. The reaction mixtures were allowed to stand for 15–30 min. at room temperature. Benzidine acetate (4 drops of 0·2 % (w/v) in 10 % (v/v) acetic acid) was then added and the intensity of the blue coloration noted. Where catalase was used 1 mg. of Kat.f. 6000 was added.

A number of extracts of different plants were tested in this way. The strongest tests were given by extracts of pea (*Pisum sativum* L.) and lupin (*Lupinus* sp.) seedlings in the presence of $MnSO_4$. Extracts of other plants gave negative tests in absence of $MnSO_4$, but in presence of $MnSO_4$ strongly positive tests were obtained with freshly prepared extracts of the leaves of barley (*Hordeum vulgare* L.), and spinach (*Beta vulgaris* L.), weaker tests were given by parsley (*Petroselinum hortense*, Hoffm.), tobacco (*Nicotiana tabacum* L.), lettuce (*Lactuca sativa* L.) and carrot seedlings (*Daucus carota* L.).

These results suggest that enzyme systems producing H_2O_2 were present in the plant extracts.

Colorimetric evidence of amine oxidation

A thermolabile fraction of the H_2O_2 producing system was isolated by precipitating pea extracts with $(NH_4)_2SO_4$, and suspending the precipitates in water. These suspensions were inactive in the benzidine tests, but could be reactivated by adding boiled extract. It was found by means of benzidine tests that the thermostable fraction passed into $CHCl_3$ from alkaline, but not from acid, extracts of pea seedlings. This suggested that the thermostable fraction was basic, and that the H_2O_2-producing enzyme might be the diamine oxidase described by Werle & Pechmann (1949) in extracts of various leguminous plants and of lavender leaves. In agreement with this it was found that the addition of putrescine reactivated the precipitate obtained with $(NH_4)_2SO_4$ in the benzidine tests and markedly increased the strength of the tests given by the pea extract. When the tests were made with the pea and lupin extracts with added putrescine in the presence of $MnSO_4$ the accumulation of manganipyrophosphate was so great as to impart a pink coloration to the reaction mixtures. But similar results were obtained when β-phenylethylamine was added in place of putrescine which suggested that the extracts oxidized mono- as well as di-amines. This was further investigated in manometric experiments. The benzidine tests given by the other plant extracts

in the presence of $MnSO_4$ were not markedly increased by adding β-phenylethylamine or putrescine. This suggests that H_2O_2 is produced by these extracts by the oxidation of substrates other than amines. Preliminary experiments indicate that one such substrate may be lactic acid.

Manometric studies of the oxidation of amines by extracts of pea seedlings

The effect of various amines on the O_2 *uptake.* The increased O_2 uptake of extracts of pea seedlings in presence of several different amines suggested that an amine oxidase was present in the extracts (Table 1). The amines most readily oxidized were the diamines putrescine, cadaverine, agmatine, 1:6-diaminohexane and histamine, and the phenylalkylamines β-phenylethylamine, di-β-phenylethylamine, tyramine, and adrenaline, and tryptamine. The oxidation of tyramine and adrenaline was not due to tyrosinase or polyphenol oxidase since neither tyrosine nor catechol were oxidized under the same conditions. The oxidation of β-phenylethylamine and di-β-phenylethylamine differs from that of the diamines in having a short lag period. This is shown in Fig. 1, where the increased O_2 uptake due to the addition of amines to pea-seedling extract is plotted against time. The aliphatic monoamines were not readily attacked, but some increase in O_2 uptake was obtained with colamine, ethylamine, allylamine and *n*-heptylamine. Of the amino-acids tested L-lysine gave a small but definite increase in the rate of O_2 uptake. These results were obtained in a number of experiments using different extracts, and therefore the increased O_2 uptake observed only provides an approximate estimate of the relative rates of oxidation at pH 7. Furthermore, the optimum pH for oxidation varies with the different amines. It is clear, however, that the

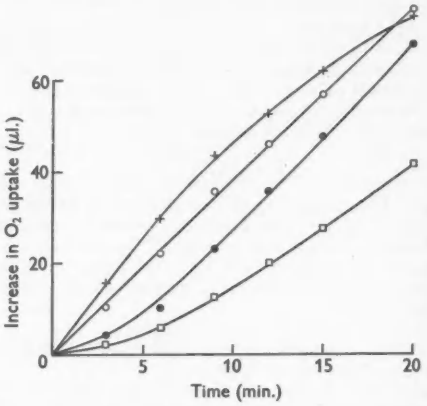


Fig. 1. The initial course of oxidation of mono- and di-amines by pea-seedling extract. Amine concentration, 0·01 M.; orthophosphate, 0·067 M. +—+, cadaverine, 0·5 ml. extract, pH 6; ○—○, putrescine, 0·5 ml. extract, pH 7·5; ●—●, di-β-phenylethylamine, 1 ml. extract, pH 8·5; □—□, β-phenylethylamine, 1 ml. extract, pH 8·5.

Table 1. *Effect of various amines on the* O_2 *uptake of extracts of pea seedlings*

(Reaction mixtures consisted of 1 ml. tissue extract (5- to 6-day old pea seedlings) in 0·067 M-orthophosphate at pH 7. The substrates, 0·3 ml. 0·1 M were added from the side arm as solutions of hydrochlorides except where otherwise stated. Uptake by extract in absence of added substrate 20–30 μl. O_2/hr.)

Substrate added	Increased O_2 uptake (μl./hr.)	Substrate added	Increased O_2 uptake (μl./hr.)
Methylamine	5	Ephedrine	<5
Ethylamine	27	Colamine	24
n-Butylamine	6	Choline	<5
n-Amylamine	10	Ethylenediamine	15
*iso*Amylamine	5	1:3-Diaminopropane	<5
n-Heptylamine	39	Putrescine	342
Allylamine	31	Cadaverine	378
*cyclo*Hexylamine	<5	1:6-Diaminohexane	150
Aniline*	<5	Agmatine†	190
Benzylamine	30	Spermine	29
β-Phenylethylamine	134	Histamine	101
β-Phenyl-β-hydroxyethylamine	9	DL-Ornithine‡	<5
Di-β-phenylethylamine	133	L-Lysine	24
Dimethylamine	<5	L-Alanine	<5
Trimethylamine	10	L-Tyrosine§	<5
Hordenine†	<5	L-Tryptophan	<5
Tyramine	54	Guanine§	<5
Tryptamine	49	Adenine§	<5
Adrenaline	54	D-Glucosamine	5

* Added as suspension of free base.
† Added as sulphate.
‡ Added as hydrobromide.
§ Added as suspension.

oxidation of the monoamines is not due to the plant monoamine oxidase described by Werle & Roewer (1950) in *Colchicum bornmulleri* Freyn., *Salvia uliginosa* Benth., *Cannabis sativa* L. var. indica Lam. and *Momordica balsamina* L., which oxidizes butylamine and methylamine more readily than tyramine and tryptamine. With the diamines, in contrast to the present results, Werle & Pechmann (1949) found that, at pH 7, putrescine was oxidized much more readily than cadaverine by extracts of lavender leaves.

Effect of pH. The variation of activity with pH was tested in 0·067M-orthophosphate, using 0·01M-β-phenylethylamine, 1:3-diaminopropane, putrescine, cadaverine and 1:6-diaminohexane. Extracts of 7- to 13-day old pea seedlings were added from the side arm after equilibration. The initial pH was determined using separate reaction mixtures and the final pH with the experimental reaction mixtures. In no case was any appreciable change of pH observed.

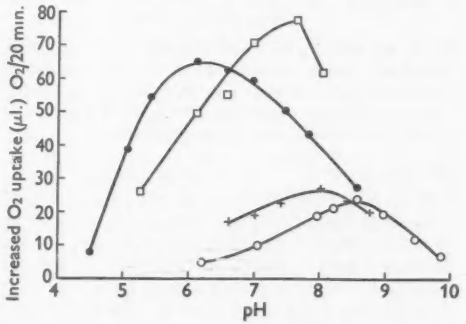


Fig. 2. Effect of pH of the medium on the rate of oxidation of amines by pea-seedling extract. Amine concentration, 0·01M; orthophosphate, 0·067M. ●—●, cadaverine; □—□, putrescine; +—+, 1:6-diaminohexane; ○—○, β-phenylethylamine.

In Fig. 2 the O_2 uptake in 20 min. is plotted against pH. The results are plotted in this way rather than as velocities, since with β-phenylethylamine there is an initial lag in the O_2 uptake (Fig. 1).

With the series of diamines the pH optima and the activity were dependent on the length of the methylene chain. Activity was negligible with 1:3-diaminopropane, optimal with putrescine (1:4-diaminopentane), decreased slightly with cadaverine (1:5-diaminopentane) and markedly with 1:6-diaminohexane.

The optimum pH was about 7·5 for putrescine, 6·0 for cadaverine, and 8 for 1:6-diaminohexane; thus the optimum varied, but not in a regular manner, with increase in methylene chain. The possibility that the differences in pH optimum were related to differences in ionization of the amines was investigated. Titration curves of the three amines up to pH 9·7 were very similar and showed that below about pH 8 the amines were completely ionized. Bredig (1894) found for putrescine $K = 5{\cdot}1 \times 10^{-4}$, and for cadaverine $7{\cdot}3 \times 10^{-4}$ at 25°. Alles & Heegard (1943) with the monoamine oxidase of animal tissues, using two series of monoamines, found varying pH optima which could not be related to the ionization constants of the amines. It is of interest that the pH optimum for the action of animal amine oxidase on β-phenylethylamine is pH 6 (Alles & Heegard, 1943), whereas with pea amine oxidase it is about pH 8·5. Under optimum conditions of pH this monoamine is attacked much less readily than putrescine and cadaverine by pea amine oxidase.

Effect of substrate concentration. The effect of substrate concentration on the initial reaction

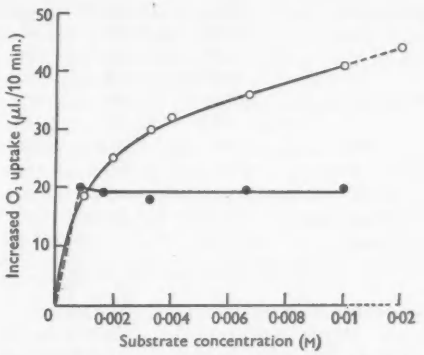


Fig. 3. Effect of substrate concentration on the rate of oxidation. ○—○, β-Phenylethylamine, 0·067M-orthophosphate pH 8·5, 1 ml. pea-seedling extract; ●—●, putrescine, 0·067M-orthophosphate pH 7·5, 0·1 ml. pea-seedling extract.

velocity was tested with putrescine and β-phenylethylamine in 0·067M-orthophosphate buffer at the optimum pH for the amines used (Fig. 3). Over the range 0·01–0·001M-substrate there was little change in initial velocity with the diamine used, but with β-phenylethylamine the initial velocity increased with substrate concentration, maximum velocity being reached at about 0·01M.

Effect of inhibitors. The effect of a number of inhibitors on the oxidation of β-phenylethylamine and putrescine by pea-seedling extract is shown in Table 2. At a concentration of 0·01M-cyanide, the oxidation of both mono- and di-amine was inhibited strongly; at 0·001M-cyanide oxidation of the diamine was only slightly inhibited, whereas a 60% inhibition of the monoamine was produced. Azide

at the same concentration was a much less effective inhibitor than cyanide, but semicarbazide was more effective causing 86–98 % inhibition at a concentration of 0·001M. The results with azide and semicarbazide on the oxidation of the diamine are in agreement with those obtained by Werle & Pechmann (1949). The comparative ineffectiveness of azide suggests that the inhibition observed with cyanide is not due to its property of complex formation with heavy metals, and the sensitivity towards

Table 2. *Effect of inhibitors*

(Reaction mixtures consisted of 1 ml. extract of 7- to 15-day old pea seedlings in 0·067M-orthophosphate at pH 7, 0·3 ml. of 0·1M-amine hydrochloride was added from the side arm. KOH was omitted from the centre cup in the experiment on KCN inhibition.)

Inhibitor	Concentration of inhibitor (M)	Percentage inhibition: β-Phenylethylamine	Percentage inhibition: Putrescine
KCN	0·01	90	99
	0·001	60	15
NaN_3	0·01	37	27
	0·001	9	10
Semicarbazide	0·01	100	98
	0·001	86	98
Ephedrine	0·1	15	19
*iso*Amylamine	0·1	83	62
1:3-Diaminopropane	0·1	92	97
p-Benzoquinone*	0·006	52	—
Iodoacetate	0·01	8	0

* Added as suspension 2 mg./vessel.

semicarbazide suggests that the activity of the enzyme may depend on the presence of a carbonyl group. This explanation for the inhibiting effect of semicarbazide on the oxidation of diamines by extracts of pea seedlings was also put forward by Werle & Pechmann (1949). The inhibiting effect of cyanide could then be due to the formation of cyanhydrin, as suggested by Zeller (1942) for animal diamine oxidase. *p*-Benzoquinone inhibited the oxidation of the monoamine, but with the diamine it caused an increase in the O_2 uptake probably due to coupled oxidation of the benzoquinone by peroxidase and the H_2O_2 formed during the oxidation of the amine. Iodoacetate did not inhibit even when incubated for 3 hr. with the extract before addition of the amines (Friedenwald & Herrmann, 1942).

Variation in activity with age of pea seedling. Werle & Pechmann (1949) showed that extracts of the resting seeds of leguminous plants were unable to oxidize diamines. Activity appeared on germination reaching a maximum in red clover seedlings after about 5 days of germination. In the present work the correlation of the activities towards both mono- and di-amines with the age of the pea seedlings has been investigated.

Peas sown in compost were harvested at various stages of growth, and the activity of extracts towards cadaverine and β-phenylethylamine determined. A typical set of results is given in Fig. 4 where the change in Q_{O_2} (μl. O_2/mg. dry wt./hr.) of the extracts with the age of the seedling is plotted. The seeds after soaking, but before germination, showed no activity towards either amine. A measurable activity first appeared 3–4 days after sowing.

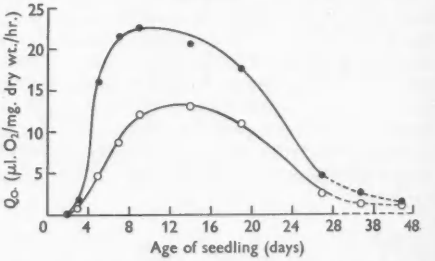


Fig. 4. Variation in rate of amine oxidation with age of pea seedling. Amine concentration 0·01M, orthophosphate concentration 0·067M. ●—●, Cadaverine, pH 6; ○—○, β-phenylethylamine, pH 8·5. The Q_{O_2} was based on the increased O_2 uptake over the first 20 min.

The activity towards both amines increased rapidly over the next few days, was maximal over the period 7–18 days, and then decreased. This decrease in activity paralleled the breakdown of the cotyledons. At later stages of growth (24–45 days) it was not possible to use cotyledon material for the extract. A slight, but measurable, activity was still present in the compost-grown seedlings at 30–45 days and also in extracts of leaves of garden pea plants at the cropping stage. The extracts from the seedlings always attacked the diamines more readily than the monoamines. This difference in the rate of attack appeared to be greatest in the first few days of activity. Although only slight activity was found in the leaves of adult pea plants, extracts of root nodules from these plants sometimes showed activity comparable with cotyledons.

Distribution of activity. In the early stages of growth (8-day old seedlings) the activity was mainly confined to the cotyledon and shoot, the root showing only a weak activity (Table 3). At a later stage (16-day old seedlings) when sufficient leaves had developed to enable differentiation between stem and leaf, activity was found in leaf, stem and root, though this was low in comparison with that of the cotyledon. In view of the fact that the cotyledons were so much more active than the other parts of the seedlings a few experiments were made with pea seedlings grown aseptically. These

Table 3. *Distribution of amine oxidase in pea seedlings*

(The activity of extracts of different parts of pea seedlings was tested manometrically. Reaction mixtures consisted of 0·2–1·5 ml. tissue extracts in 0·067 M-orthophosphate at pH 6 (cadaverine) or 8·5 (β-phenylethylamine) and 0·3 ml. 0·1 M-amine hydrochloride added from the side arm. The Q_{O_2} and activity per ml. extract was based on the increase in O_2 uptake over the first 20 min.)

	8-day seedlings					16-day seedlings				
		β-Phenylethylamine		Cadaverine			β-Phenylethylamine		Cadaverine	
Part	Average wet wt. (g./seedling)	Increased O_2 uptake (μl./ml. extract/hr.)	Q_{O_2}	Increased O_2 uptake (μl./ml. extract/hr.)	Q_{O_2}	Average wet wt. (g./seedling)	Increased O_2 uptake (μl./ml. extract/hr.)	Q_{O_2}	Increased O_2 uptake (μl./ml. extract/hr.)	Q_{O_2}
Root	0·16	33	2	91	5	0·58	15	3	21	4
Shoot	0·12	184	11	264	14	—	—	—	—	—
Cotyledon	0·64	870	16	1680	32	0·64	1086	46	2331	97
Leaf	—	—	—	—	—	0·44	81	2	151	4
Stem	—	—	—	—	—	0·45	42	2	84	5

seedlings proved to be as active towards amines as those grown in compost and it is therefore clear that the high activity of the cotyledons was not due to infection.

Results with other plants

Extracts of leaves of lavender (*Lavendula spica* Cav.) and of red clover (*Trifolium pratense* L.) and of seedlings of garden lupins (*Lupinus hybridus* Hort.) and of tree lupins (*L. arboreus* L.), shown by Werle & Pechmann (1949) to oxidize diamines, were tested manometrically with putrescine and β-phenylethylamine. In every case oxidation of both mono- and di-amine was found, the diamine always being oxidized more readily than the monoamine. Neither the mono- nor the di-amine were oxidized by extracts of spinach (*Beta vulgaris* L.), marrow (*Cucurbita pepo* L.), lettuce (*Lactuca sativa* L.), barley (*Hordeum vulgare* L.), parsley (*Petroselinum hortense* Hoffm.), and tobacco (*Nicotiana tabacum* L.).

The course of the oxidation

A quantitative study has been made of the total O_2 uptake during the oxidation of the amines and of the products of oxidation. The results obtained depended on whether the extract or the dry preparation of enzyme was used to catalyse the reaction. In these experiments β-phenylethylamine and putrescine were used as representatives of the mono- and di-amines attacked. The results suggest that the primary attack on the amines is an oxidative deamination according to the equation:

$$R.CH_2.NH_2 + H_2O + O_2 \rightarrow R.CHO + H_2O_2 + NH_3. \quad (1)$$

If the peroxide formed is decomposed by catalase the net reaction is

$$R.CH_2.NH_2 + H_2O + \tfrac{1}{2}O_2 \rightarrow R.CHO + H_2O + NH_3. \quad (2)$$

Hydrogen peroxide formation

Some evidence that H_2O_2 is formed during the oxidation of diamines by extracts of pea seedlings was obtained by Werle & Pechmann (1949). Using the cerous hydroxide method they were able to demonstrate 20 % of the H_2O_2 required by Eqn. 1. In the present work the formation of H_2O_2 during the oxidation of the amines was shown, with the dry preparation, by the effect of catalase on the total O_2 uptake, and, with the extract, by the coupled oxidation of the phenols, quinol and *p*-cresol, through peroxidase present in the extract (Thurlow, 1925; Stephenson, 1943), and also of ethanol in the presence of added catalase (Keilin & Hartree, 1936, 1945; Philpot, 1937; Kohn, 1937).

Dry preparation. Using the dry preparation in the presence of added catalase with putrescine as substrate the total O_2 uptake corresponded to 1 atom O_2/mol. amine, in accordance with Eqn. 2. In absence of added catalase the total uptake approached 1 mol. O_2/mol. amine (Eqn. 1) and the rapid uptake was succeeded by a slow output. This increased uptake in absence of added catalase and the subsequent slow output were due to the accumulation and slow decomposition of H_2O_2 since, on addition of catalase from the side arm of the manometer vessel, a rapid output of gas took place causing a drop in the overall uptake from 1 mol. to 1 atom O_2/mol. amine (Fig. 5*a*). Tabor (1951) has demonstrated H_2O_2 formation by animal diamine oxidase in the same way. The results obtained with β-phenylethylamine as substrate were similar except that the total O_2 uptake in presence of added catalase was greater than 1 atom O_2/mol. amine, and the difference in uptake in presence and absence of added catalase was therefore not as great as with putrescine as substrate (Fig. 5*b*).

In these experiments the reaction mixtures consisted of 0·0025 M-putrescine or β-phenylethylamine

in 0·067 M-orthophosphate at pH 7·5 and 8·5 respectively, 2·5 mg. dry preparation and 0·1 mg. catalase (Kat.f. 6000, where present). With the dry preparation with β-phenylethylamine as substrate some coupled oxidation of ethanol was obtained in presence of added catalase, though the increase in O_2 uptake was not as great as with putrescine as substrate.

oxidation either of ethanol in the presence of added catalase or of quinol and *p*-cresol (Tables 4 and 5). With β-phenylethylamine as substrate the formation of H_2O_2 could not be demonstrated by either of these methods. Since β-phenylethylamine is attacked much less rapidly than putrescine, much larger amounts of extract are required to produce a suitable rate of oxidation. Quantitative studies on

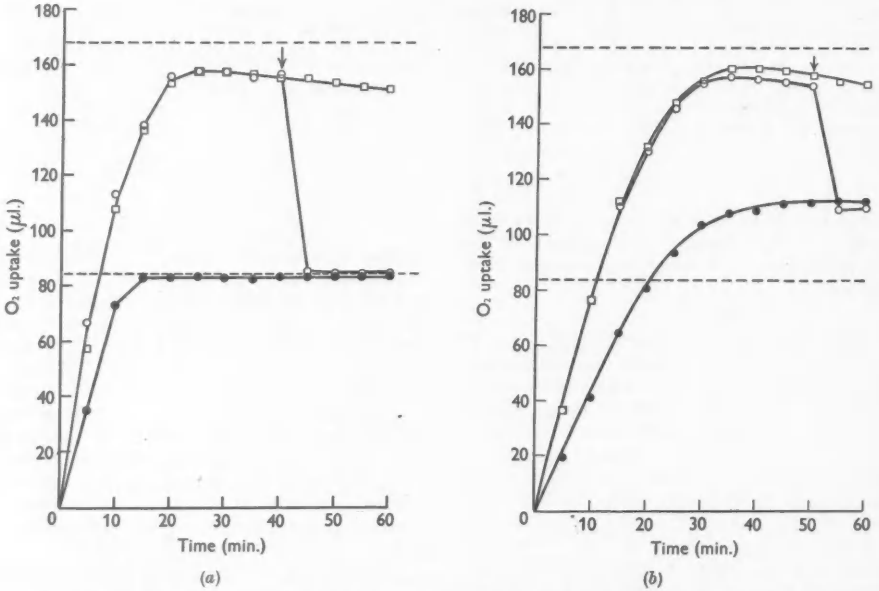


Fig. 5. Formation of hydrogen peroxide during the oxidation of putrescine and β-phenylethylamine by the amine oxidase preparation. Amine concentration, 0·0025 M; orthophosphate, 0·067 M. Catalase 0·1 mg. Kat.f. 6000 (where present). (*a*) Putrescine; (*b*) β-phenylethylamine. □—□, No catalase; ○—○, catalase added at ↓; ●—●, catalase added at zero time. The dotted lines show the uptake for 1 atom and 1 molecule of oxygen/molecule amine.

Table 4. *Coupled oxidation of phenols during the oxidation of putrescine by extracts of pea seedlings*

(Reaction mixtures consisted of 0·2 ml. extract of 6-day old pea seedlings in 0·067 M-orthophosphate at pH 6·5, 0·2 ml. of 0·02 M-quinol or *p*-cresol, and 0·2 ml. of 0·025 M-putrescine added from the side arm.)

Reaction system	Total O_2 uptake (μl.)
Extract	4
Extract + quinol or *p*-cresol	2
Extract + putrescine	66
Extract + putrescine + quinol	114
Extract + putrescine + *p*-cresol	117

Table 5. *Coupled oxidation of ethanol during the oxidation of putrescine by extracts of pea seedlings*

(Reaction mixtures consisted of 10 mg. catalase (Kat.f. 6000), 5 mg. ethanol, 0·4 ml. of 0·025 M-putrescine in 0·067 M-orthophosphate at pH 7·5. 0·2 ml. extract of 7-day old pea seedlings was added from the side arm.)

Reaction system	Total O_2 uptake (μl.)
Extract	10
Extract + putrescine	126
Extract + putrescine + catalase	145
Extract + putrescine + ethanol	126
Extract + putrescine + ethanol + catalase	231

Extract. When the extract is used to catalyse the oxidation of the amines, H_2O_2 does not accumulate. With putrescine as substrate, however, the formation of H_2O_2 can be demonstrated by the coupled

the O_2 uptake suggest that under these conditions the H_2O_2 formed is used for the coupled oxidation of substrates present in the extract. Quinol and *p*-cresol inhibited the oxidation of β-phenyl-

Table 6. *Oxygen uptake and aldehyde and ammonia formation during the oxidation of β-phenylethylamine and putrescine*

(Reaction mixtures consisted of extract of 8- to 12-day old pea seedlings, or dry preparation, in 0·067 M-orthophosphate at pH 7·5 (putrescine) or 8·5 (β-phenylethylamine). Catalase 0·1 mg. (Kat.f. 6000) was added where the dry preparation was used. Amine hydrochloride was added from the side arm.)

Enzyme	Amine added (μmol.)	Total O_2 uptake (μmol.)	Aldehyde formed (μmol.)	Ammonia formed (μmol.)
	β-Phenylethylamine			
Extract 1·5 ml.	5	11·3	1·1	5·8
	7·5	16·4	3·0	7·4
	10	20·5	4·5	10·5
Dry preparation 10 mg.	5	3·2	3·8	5·4
	7·5	5·2	5·6	8·3
	10	7·2	7·8	9·9
	Putrescine			
Extract 0·2 ml.	5	3·1	4·6	5·9
	7·5	4·2	6·8	8·8
	10	5·4	8·5	11·8
Dry preparation 5 mg.	5	2·5	4·8	5·4
	7·5	3·7	6·8	7·5
	10	5·1	8·6	11·2

ethylamine by the extract, possibly owing to the formation of quinones by coupled oxidation. As shown earlier (Table 2), *p*-benzoquinone inhibits the oxidation of β-phenylethylamine.

Total oxygen uptake, aldehyde and ammonia formation

The total O_2 uptakes, and the amounts of aldehyde and NH_3 formed during the oxidation of putrescine and β-phenylethylamine by pea amine oxidase are shown in Table 6.

Putrescine

With putrescine as substrate using 0·2 ml. of extract the O_2 uptake reached completion in about 90 min. and corresponded to rather more than 1 atom O_2/mol. amine. Werle & Pechmann (1949), using extracts of pea or clover seedlings with cadaverine as substrate, found O_2 uptakes of about 1 mol. O_2/mol. amine. In their experiments larger amounts of more concentrated seedling extracts were used and it is probable that the greater O_2 uptakes observed were due to the H_2O_2 formed being used in secondary oxidations. Using 5 mg. of the dry preparation together with added catalase in place of the extract the total O_2 uptake corresponded more closely to 1 atom O_2/mol. amine. In both cases the values found for aldehyde and NH_3 were in the neighbourhood of 1 mol./mol. amine, the values for NH_3 being slightly higher than this, particularly where the extract was used, and those for aldehydes slightly lower. It therefore appears that the reaction proceeds according to Eqn. 2 and as with animal diamine oxidase (Zeller, 1942) only one of the $-NH_2$ groups of the diamine is attacked. Tabor (1951), with animal diamine oxidase, was unable to use the bisulphite method since the reaction products (particularly that of cadaverine) reacted with I_2 even in absence of bisulphite. In the present work using putrescine, experiments showed that the reaction products did not react directly with I_2 sufficiently rapidly to affect the results of the bisulphite method.

β-Phenylethylamine

The total O_2 uptake with β-phenylethylamine as substrate using the dry preparation in presence of added catalase varied between 1·2 and 1·5 atoms O_2/mol. amine. The amounts of NH_3 found were in agreement with Eqn. 2, but the aldehyde values were somewhat low. With the extract it was necessary to use at least 1·5 ml. in order that the increased O_2 uptake in presence of β-phenylethylamine should be complete within 3 hr. Under these conditions the O_2 uptake was rather more than 2 mol. O_2/mol. amine. The NH_3 produced corresponded to complete deamination of the amine, but the amounts of aldehyde as estimated by bisulphite titration were low, varying between 22 and 45 % of theory. The bisulphite titration was unsatisfactory with these large amounts of extract as the end point was indefinite. The fact that the amounts of aldehyde found were so low suggested that the extra O_2 uptake was in fact due to further oxidation of the phenylacetaldehyde formed as the primary oxidation product. It was found that large increases in the O_2 uptake were produced on the addition of phenylacetaldehyde to pea-seedling extract or to the dry preparation, presumably owing to oxidation of the aldehyde to phenylacetic acid. The total increase in O_2 uptake was, however, insufficient to account for the oxidation to the corresponding acid

of all the aldehyde added. No increase in O_2 uptake was produced by the addition of acetaldehyde, benzaldehyde and a number of other aldehydes to the extracts or dry preparations. Preliminary tests suggest that the oxidation of phenylacetaldehyde is accompanied with H_2O_2 formation. The oxidation of β-phenylethylamine to the corresponding aldehyde would require 2 atoms of O_2 if the H_2O_2 formed was used in secondary oxidations. Similarly, further oxidation of the aldehyde to phenylacetic acid would require a further 2 atoms of O_2 giving a total uptake of 4 O_2 atoms/mol. amine which is in close agreement with that obtained experimentally with the extract. According to the values obtained by bisulphite titration, however, the aldehyde is not completely oxidized. With the dry preparation the O_2 uptake in excess of 1 mol. O_2/mol. amine can be accounted for by further oxidation of aldehyde as shown by the bisulphite estimations. It is not yet clear, however, why the oxidation, particularly with the enzyme preparation, stops when large amounts of aldehyde are still present.

Isolation of phenylacetaldehyde 2:4-*dinitrophenylhydrazone*. Using the dry preparation it has been proved that phenylacetaldehyde is a primary product of the oxidation of β-phenylethylamine by its isolation as the 2:4-dinitrophenylhydrazone.

A reaction mixture containing 10 ml. 0·1 M-β-phenylethylamine, 500 mg. dry preparation, 10 mg. catalase (Kat.f. 6000) and 100 ml. 0·2 M-orthophosphate, pH 8·5, in a total volume of 300 ml. was shaken in air at room temperature for 4 hr. The oxidation was followed by estimating the aldehyde present in 2 ml. samples taken at half-hourly intervals. Aldehyde was rapidly formed during the first hour at the end of which the amount present was 50% of the theoretical. Thereafter the increase was slow, and had virtually ceased at 4 hr., when the amount present was 75% of the theoretical. The residual solution (284 ml.) which had the characteristic hyacinth odour of phenylacetaldehyde, was brought to pH 2 with 3 N-HCl and centrifuged. To the centrifugate 80 ml. of a saturated solution of 2:4-dinitrophenylhydrazine in 2 N-HCl was added, and the mixture was allowed to stand overnight at room temperature. The yellow precipitate was then centrifuged off and extracted several times with boiling ethanol. The combined filtered ethanol extract was concentrated and left in the refrigerator overnight. The yellow crystals were filtered off and dried *in vacuo*. Wt. 96 mg. Yield: 35% theory based on amine added, 46·5% based on the bisulphite titration. The m.p. (uncorr.) was 111·5–113·5°. After recrystallization from ethanol the m.p. (uncorr.) was 116·5–117·5°, unchanged on mixture with an authentic sample (m.p. (uncorr.) 118–119°) prepared according to Campbell (1936).

DISCUSSION

The results of the colorimetric tests described in the present work suggest that hydrogen peroxide is produced by enzyme systems present in extracts of many plants, and that this hydrogen peroxide can be used by the extracts for the oxidation of added Mn^{++}. Since under certain conditions the oxidized manganese accumulates and gives a blue coloration with benzidine, it is frequently possible by addition of Mn^{++} to demonstrate hydrogen peroxide formation in extracts which give negative tests in absence of added Mn^{++}.

Attempts to identify these systems have so far shown that both mono- and di-amines are oxidized by extracts of pea seedlings with the production of hydrogen peroxide. Of the monoamines tested those most readily attacked are mono- and di-β-phenylethylamine, tyramine and tryptamine, while the aliphatic monoamines such as methylamine and butylamine are only feebly attacked. The plant monoamine oxidase described by Werle & Roewer (1950) oxidizes methylamine and butylamine more readily than tyramine and tryptamine. With the animal monoamine oxidase tyramine, tryptamine and *iso*amylamine are most readily attacked (Blaschko, Richter & Schlossmann, 1937), though the relative rates at which these are attacked differ with different tissues and species (Blaschko, 1941; Alles & Heegard, 1943). The latter two enzymes are not inhibited by 0·001 M-cyanide, which causes a 60% inhibition of the oxidation of monoamines by pea-seedling extracts. It appears, therefore, that the pea-seedling enzyme which attacks monoamines differs from the previously described plant and animal monoamine oxidases.

Of the diamines tested no activity is observed with 1:3-diaminopropane; putrescine and cadaverine are rapidly attacked, at about the same rate, and 1:6-diaminohexane is much less readily attacked. With animal diamine oxidase, using the $\alpha\omega$ series of diamines the rate of oxidation increases with the length of the methylene chain up to five methylene groups (Zeller, 1942). The available evidence suggests that the oxidation of both mono- and di-amines by the plant extracts used in the present work may be brought about by the same enzyme. The plant extracts which oxidized diamines also oxidized monoamines and the relative rates of attack on the two types of amine were similar. Both activities appear at about the same time in germinating pea and lupin seedlings and are, in general, affected in the same way by inhibitors. As with animal diamine oxidase, and in contrast to animal monoamine oxidase, both cyanide and semicarbazide inhibit the action though the oxidation of the diamines, in particular, is less sensitive to cyanide than is the case with animal diamine oxidase. Amines which are not themselves attacked inhibit the oxidation of both mono- and di-amines to the same extent. Of the amines tested in this way the diamine 1:3-diaminopropane is the strongest inhibitor, while ephedrine, which is well known to inhibit animal monoamine oxidase, is a much less effective inhibitor than *iso*amylamine.

While the above findings suggest that the oxidation of both mono- and di-amines is catalysed by one enzyme the fact that the oxidation of monoamines, but not that of diamines, is inhibited by *p*-benzoquinone suggests the possibility that specific mono- and di-amine oxidases may be present in the plant. Further evidence is necessary to distinguish between these two possibilities. Until such evidence has been obtained it appears preferable to refer to the enzyme as plant amine oxidase rather than diamine oxidase even though the diamines are more readily attacked than the monoamines.

As with the amine oxidases of animal tissues the primary attack on the amines consists of an oxidative deamination according to the equation

$$R.CH_2NH_2 + H_2O + O_2 \rightarrow R.CHO + H_2O_2 + NH_3.$$

The formation of hydrogen peroxide during the oxidation suggests that plant amine oxidase is a flavoprotein. It seems of particular interest that tryptamine is among the amines attacked by the enzyme. According to Skoog (1937) and Gordon & Nieva (1949), tryptamine can function as a precursor of 3-indolylacetic acid in plants. The action of the enzyme on tryptamine presumably leads to the formation of 3-indolylacetaldehyde. It has already been mentioned that an enzyme is present in pea-seedling extracts which oxidizes phenylacetaldehyde. Preliminary experiments suggest that this enzyme is widely distributed among higher plants. It is possible that 3-indolylacetaldehyde is one of its substrates and that 3-indolylacetic acid could be formed from tryptamine by the action of amine oxidase followed by that of the aldehyde oxidase. The hypothesis has been advanced that the herbicidal activity of 2-phenoxyethylamine derivatives is due to their conversion to derivatives of 2-phenoxyacetic acid by the living plant (Jones, Metcalfe & Sexton, 1949).

SUMMARY

1. A colorimetric test, based on the oxidation of added Mn^{++} to Mn^{+++}, is described for the detection of hydrogen peroxide in plant extracts. By means of this test evidence has been obtained that hydrogen peroxide is produced by enzyme systems present in extracts of many higher plants.

2. The oxidation of both mono- and di-amines is catalysed by extracts of pea and lupin seedlings and leaves of lavender and red clover. It is not yet known whether this is due to the presence of specific mono- and di-amine oxidases.

3. With the growth conditions used, activity appears in pea seedlings, mainly in the cotyledons, 3–4 days after germination and is maximal over the period 7–18 days. Only slight activity is found in the adult plant.

4. Diamines, in particular putrescine and cadaverine, are more readily attacked than monoamines. Of the monoamines tested β-phenylethylamine and di-β-phenylethylamine are most readily attacked.

5. The oxidative deamination of β-phenylethylamine and putrescine is catalysed according to the equation

$$R.CH_2NH_2 + O_2 + H_2O \rightarrow R.CHO + H_2O_2 + NH_3.$$

As with animal diamine oxidase only one of the terminal amino groups is attacked.

6. The phenylacetaldehyde formed by the action of the plant amine oxidase on β-phenylethylamine was oxidized by an aldehyde oxidase present in the preparation used.

7. It is suggested that 3-indolylacetic acid may be formed by the successive action of the plant amine oxidase and of the aldehyde oxidase on tryptamine.

The authors wish to thank Dr J. Kleczkowska and Dr P. S. Nutman for help in growing pea seedlings under sterile conditions.

REFERENCES

Agner, K. (1938). *Biochem. J.* **32**, 1702.
Alles, G. A. & Heegard, E. V. (1943). *J. biol. Chem.* **147**, 487.
Blaschko, H. (1941). *J. Physiol.* **99**, 364.
Blaschko, H., Richter, D. & Schlossmann, H. (1937). *Biochem. J.* **31**, 2187.
Bredig, G. (1894). *Z. physiol. Chem.* **13**, 289.
Campbell, N. R. (1936). *Analyst*, **61**, 391.
Clift, F. P. & Cook, R. P. (1932). *Biochem. J.* **26**, 1788.
Friedenwald, J. S. & Herrmann, H. (1942). *J. biol. Chem.* **146**, 411.
Goldacre, P. L. (1949). Private communication.
Gordon, S. A. & Nieva, F. S. (1949). *Arch. Biochem.* **20**, 367.
Jones, R. L., Metcalfe, T. P. & Sexton, W. A. (1949). *Biochem. J.* **45**, 143.
Keilin, D. & Hartree, E. F. (1936). *Proc. roy. Soc.* B, **119**, 141.
Keilin, D. & Hartree, E. F. (1945). *Biochem. J.* **39**, 293.
Kenten, R. H. & Mann, P. J. G. (1949). *Biochem. J.* **45**, 255.
Kenten, R. H. & Mann, P. J. G. (1950). *Biochem. J.* **46**, 67.
Kohn, H. J. (1937). *Biochem. J.* **31**, 1693.
Markham, R. (1942). *Biochem. J.* **36**, 790.
Philpot, F. J. (1937). *Biochem. J.* **31**, 856.
Pugh, C. E. M. & Quastel, J. H. (1937). *Biochem. J.* **31**, 282.
Skoog, F. (1937). *J. gen. Physiol.* **20**, 311.
Stephenson, N. R. (1943). *J. biol. Chem.* **149**, 169.
Sumner, J. B. & Somers, G. F. (1943). *Chemistry and Methods of Enzymes*, p. 171. New York: Academic Press.
Tabor, H. (1951). *J. biol. Chem.* **188**, 125.
Thornton, H. G. (1930). *Ann. Bot., Lond.*, **44**, 385.
Thurlow, S. (1925). *Biochem. J.* **19**, 175.
Werle, E. & Pechmann, E. (1949). *Liebigs Ann.* **562**, 44
Werle, E. & Roewer, F. (1950). *Biochem. Z.* **320**, 298.
Zeller, E. A. (1942). *Advanc. Enzymol.* **11**, 93.

Methods of Paper Chromatography of Steroids Applicable to the Study of Steroids in Mammalian Blood and Tissues

By I. E. BUSH
National Institute for Medical Research, Mill Hill, London, N.W. 7

(*Received* 21 *April* 1951)

Chromatography has already been widely applied to the steroids, both for preparative and analytical work. Both adsorption and partition methods have been used, although the lipoidal solubility properties of these compounds have made the latter method difficult to apply. Burton, Zaffaroni & Keutmann (1950) have overcome these difficulties by using filter paper soaked in propylene glycol or formamide, and a hydrocarbon mobile phase, with good results. Earlier work with steroid Girard derivatives only gave separations according to the number of ketone groups available for condensation with Girard's reagent (Zaffaroni, Burton & Keutmann, 1949).

The present paper describes some of the results obtained during an attempt to devise paper chromatographic methods suitable for the analysis of steroids in small amounts in body fluids and biological media. The emphasis throughout has been on methods that are reasonably quick and simple, so as to be suitable for routine analytical work. In large measure these ends have been achieved, though as yet the methods described have only been used semi-quantitatively. Two types of system will be described: (1) an adsorption method using alumina-impregnated paper (Datta, Overell & Stack-Dunne, 1950; Bush, 1950*a*); (2) partition systems using aqueous methanol as stationary phase. Special methods have been developed for preparing the extracts from biological material and applying them to the papers and these are also described.

METHODS

Chromatography on alumina paper

Preparation of papers. Sheets of 20 × 45 cm. Whatman no. 54 or no. 4 were immersed for 5–10 sec. in a solution of $Al_2(SO_4)_3$ (A.R., 300 g./l.) made just cloudy with 2N-NaOH, at 60–70°, taken out, hung double over glass rods and suspended vertically in a large glass tank. The lids were immediately closed and 15 min. allowed to elapse. NH_3 solution (sp.gr. 0·880) was then pumped into the tank to a depth of 4–6 cm. by way of a tube and rubber bung near the bottom of the tank. The papers were left hanging in the vapour for 18 hr. On removal they were placed in another tank and immersed in about 25 l. of a solution of $CaCl_2$ (Becker's fused granulated $CaCl_2$, 0·2 g./l.). This product contains much lime and gives an alkaline solution. The solution was continually aspirated with a stream of air to remove excess NH_3. The papers were removed after 24 hr. to dry at room temperature.

Solvents. The papers were run in glass cylinders by the 'ascending' technique, usually to a height of 22–30 cm. The solvents used were benzene (A.R.), benzene-$CHCl_3$ (4:1, 3:1, 2:1), and 5% (v/v) acetone in benzene.

Detection of spots. After thorough drying the chromatograms were dipped quickly into a saturated solution of iodine in light petroleum (b.p. 60–80°). After drying, the background iodine sublimed and spots of steroid showed up yellow or brown on a faintly coloured background.

This reaction is evanescent, non-specific (Brante, 1949) and repeatable. It is prevented or greatly reduced in sensitivity by traces of water, organic solvents, or acid. For permanent records reflex photography in ultraviolet light, or spraying with a concentrated starch solution was used.

pH *of papers.* All papers were tested before use by dabbing with 'Universal' indicator (British Drug Houses Ltd.). When just dry the colour given corresponded with a pH of 7·0–7·5. Papers not treated with the $CaCl_2$ solution are very acid and of low adsorptive activity. Graded activity can be obtained by varying the concentration of $CaCl_2$ used.

This procedure was the routine one evolved from many variants. It was used for the detection of 17-hydroxycorticosterone in adrenal venous blood (Bush, 1950*b*), the examination of adrenal extracts, and of a number of blood samples from sheep, horse, and human subjects. It gave the most reproducible results and optimum sensitivity to the iodine reaction.

The activation with alkaline calcium chloride solution (other solutions such as 0·05N aqueous ammonia or very hard tap water, can also be used) was the key to reproducible results. Without this treatment the activity was low, R_F values varied greatly with the amount of steroid present, and some irreversible adsorption occurred at the origin of the chromatogram. After activation R_F values varied only slightly with amount of steroid and there was no residue at the origin.

The form of the spots varied. Oestrone and oestradiol gave symmetrical spots as on a partition chromatogram: an ion-exchange mechanism is probable for these phenolic compounds. The other steroids gave sharp-fronted, comet-shaped spots, with small tails.

Cortical steroids must be acetylated before flowing on alumina (Reichstein & Shoppee, 1949).

Table 1. *R_F values of various steroids on activated alumina paper*

(These values are taken from medium activity papers with a surface reaction to 'Universal' indicator (British Drug Houses Ltd.) between pH 7·5 and 7·8. Solvents: (*a*) benzene + $CHCl_3$, 3:1 (by vol.); (*b*) benzene + $CHCl_3$, 2:1; (*c*) benzene + $CHCl_3$, 1:1; (*d*) benzene + acetone, 19:1.)

Compounds	Solvents			
	(*a*)	(*b*)	(*c*)	(*d*)
Progesterone	0·75	0·95	1·0	0·8
Testosterone	0·40	0·60	—	—
Oestrone	0·30	0·40	0·9	0·6
Oestradiol	0·05	0·15	0·5	0·25
Pregnenolone	—	0·80	—	—
Dehydroandrosterone	—	0·41	—	—
Androsterone	0·25	0·39	—	—
11-Deoxycorticosterone 21-acetate	0·70	0·85	—	0·70
11-Dehydrocorticosterone 21-acetate	0·35	0·60	0·95	0·40
11-Dehydro-17-hydroxycorticosterone 21-acetate	0·05	0·10	0·40	0·10
17-Hydroxycorticosterone 21-acetate	0·02	0·06	0·30	0·05
Corticosterone 21-acetate	—	0·15	0·67	0·20

They are then quite stable. Although the acetates of 11-dehydro-17-hydroxycorticosterone and 17-hydroxycorticosterone flow with different R_F values they are only separated by long flows in benzene-chloroform (3:1) or 5% acetone in benzene. R_F values are given in Table 1.

The iodine reaction gave very good results on alumina papers just alkaline to neutral, but not on acid papers or plain filter paper. Prolonged immersion and warming the iodine solution to 35° were necessary for the latter. On the standard, slightly alkaline, alumina papers, the lower limit of sensitivity was about 0·5 μg./sq.cm. for the two oestrogens, and 1 μg./sq.cm. for a variety of $\alpha\beta$-unsaturated ketosteroids. The reaction with neutral steroids lacking the $\alpha\beta$-unsaturated ketone group was much weaker.

This reaction is similar to Brante's reaction for nitrogenous compounds (Brante, 1949), but his explanation is difficult to apply to the steroids. However, the fact that traces of moisture and other solvents remaining in the paper can almost completely stop the colour reaction, and that the colours given are what might be expected from iodine in solution in the steroid, suggests that the 'reaction' is simply a partition of the iodine between petroleum and steroid, the latter taking up an appreciable amount of iodine from the saturated solution. Estimation of the iodine taken up by steroid spots showed that four to seven atoms of iodine could be held per steroid molecule. However, the rapid evaporation of the iodine did not make this a suitable method of estimating the steroids quantitatively.

One peculiar phenomenon is not explained by this simple theory. 11-Dehydro-17-hydroxycorticosterone (cf. Burton, Zaffaroni & Keutmann, 1950) and 11-deoxy-17-hydroxycorticosterone give a yellow colour on alkaline papers (pH about 7·5), green on neutral papers (pH, 7·0), and a bright blue on acid papers (pH about 6·5). The 21-acetates behave similarly. All other steroids tried gave colours in the range yellow to red-brown.

This method can give useful separations with short quick flows (15–40 cm.) and simple apparatus, with maximum sensitivity of the iodine reaction. It has several disadvantages, however, inherent in an adsorption method, which preclude its use in many investigations. In the first place, R_F values still show a slight variation with amount of steroid, even on papers of high activity and, in the second, all extracts of biological materials must be carefully purified before chromatography, otherwise impurities may produce disastrous displacement effects. These disadvantages have been overcome in the partition methods described below.

Paper partition systems for steroids

With all common organic solvents water is not a satisfactory stationary phase for steroid chromatography. The steroids move either with the solvent front, or as streaks. However, the streaky chromatograms were greatly improved by adding small amounts of *iso*propanol to the system (hydrocarbon/water). In view of this it seemed worth while to try to find systems using hydrocarbon solvents and aqueous methanol (Cornish, Archibald, Murphy & Evans, 1934; Butt, Morris & Morris, 1949) which would work on paper, particularly since the type of mobile phase used flows very quickly on filter paper.

The ordinary 'descending' technique is used, and only those points peculiar to the present method are described.

Apparatus. All tanks were enclosed in a thermostatically controlled box and maintained while in use at an elevated temperature, usually between 30 and 38°. In order to avoid the worst effects of current variation in mains supply, three-quarters of the heating elements (in terms of power) were run through a Simmerstat (J. W. Towers and Co. Ltd., Widnes) adjusted to keep the box 1–2° below the desired

temperature. The thermostat (Sunvic TS3 air type, Sunvic Controls Ltd., London, W.C. 2) was set to the desired temperature and controlled the remaining heaters. The air in the box was circulated by a fan suspended from the lid, with the motor outside and the shaft passing through the lid. Tests with six simultaneously recording thermocouples showed a maximum spatial temperature difference of 0·5–1·0°. Without the fan operating this difference was usually 4–8°. Over periods of time up to 6 days the temperature could be kept within 0·5° of the mean.

Glass tanks. Owing to the great volatility of the solvents used, proper equilibration is much more difficult than with the usual chromatographic solvents. Saturation of the atmosphere is achieved by covering the walls of each tank with wads of filter paper continually soaking up the two phases used. Two wads dip into about 2 cm. depth of mobile phase covering the bottom of the tank, and a third into a small basin containing about 600 ml. stationary phase.

Hanging the chromatograms. The solvents are all fast-running, and to control the initial rush of solvent on to the paper it is essential to have the starting line at least 6 cm. from the trough edge, and to have the first 4 cm. of the sheet horizontal.

If a slower run is desired the horizontal stretch can be increased, or the trough suspended so that the first 8 cm. of the sheet rises at an angle of about 60°, before bending over a glass rod.

With the ordinary arrangement a 45 cm. flow takes 100–180 min. with the solvents used. This time can be varied at will by combination of the arrangements of the sheet described above.

Equilibration. The sheets were usually left hanging overnight at the desired temperature. With some solvents, however, this was not necessary and 3–4 hr. was quite sufficient.

Running the solvents. Solvents were left equilibrating overnight at the desired temperature and then poured into the troughs by holes and funnels in the lids of the tanks (Hanes & Isherwood, 1949).

The best results were obtained with aqueous methanol as stationary phase, although ethanol and *iso*propanol were suitable at low temperatures.

Solvent systems. The following systems have been used extensively and successfully. They were prepared by mixing the solvents at the operating temperature and allowing the two phases formed to separate. In all cases light petroleum was the fraction of b.p. 80–100°, methanol was absolute and water was distilled.

A. 1000 ml. light petroleum, 800 ml. methanol, 200 ml. water.

B. (1) 500 ml. toluene, 500 ml. light petroleum, 700 ml. methanol, 300 ml. water.
(2) 667 ml. toluene, 333 ml. light petroleum, 600 ml. methanol, 400 ml. water.
(3) 667 ml. light petroleum, 333 ml. benzene, 800 ml. methanol, 200 ml. water.
(4) 1000 ml. toluene, 500 ml. methanol, 500 ml. water.
(5) 1000 ml. benzene, 500 ml. methanol, 500 ml. water.

C. 900 ml. toluene, 100 ml. ethyl acetate, 500 ml. methanol, 500 ml. water.

Detection of spots. After running and drying, the sheets were pinned down over Ilford Reflex paper no. 50 and exposed briefly to ultraviolet light, using a lamp emitting nearly all its energy in the 254 mμ. region. With the lamp 60 cm. above the paper an exposure of 2–3 sec. was usual.

Despite the sensitivity of this photographic method, the presence in biological extracts of impurities which absorb ultraviolet light close to the 240 mμ. absorption peak of $\alpha\beta$-unsaturated ketosteroids often made it impossible to detect surely less than 10 μg. steroid/spot. This difficulty could be overcome by the following fluorescence reaction: the papers were simply sprayed with a solution of 10 g. NaOH in 100 ml. of 60% (v/v) methanol and dried at 60–100°. When quite dry the $\alpha\beta$-unsaturated ketosteroids gave a brilliant primrose-yellow fluorescence in ultraviolet light which faded after several days but could be developed again by a second treatment.

This reaction is very sensitive (2 μg./sq.cm.) and within the group of steroids examined seems to be specific for steroids with an $\alpha\beta$-unsaturated ketone grouping. Thus eleven steroids containing this group in ring *A* all gave positive reactions, whereas nine steroids with various substituent groups, but containing a 3-hydroxyl group, gave no fluorescence, except equilenin acetate and oestradiol which gave a blue fluorescence. All the active cortical steroids, and also testosterone, androst-4-ene-3:17-dione, androst-4-ene-3:11:17-trione, and 11-deoxy-17-hydroxycorticosterone gave positive reactions. The major advantage of this method is that no other compounds in natural extracts have been found to give this particular colour, so that the background colour is completely dark except for spots of blue or green fluorescence.

Other colour reactions can be used to check the nature of unknown ultraviolet-absorbing spots, but for many problems the photographic method is sufficient.

General procedure. The best results are obtained with medium or slow runs after equilibration overnight. Most of the systems, however, can be safely run after 3 hr. equilibration. A 'fast' chromatogram can thus be equilibrated, run, and examined within 5–5·5 hr. This has been done repeatedly with the last two systems of group *B*.

The stationary phase should be replaced every fortnight with fresh aqueous methanol, and with toluene the mobile phase also needs changing. Benzene and light petroleum only need replenishment.

Uses of the partition methods

The steroids so far studied have been largely those containing the $\alpha\beta$-unsaturated ketone group in ring *A*. For the purposes of chromatography they can be divided into three groups according to their polarity and solubility properties as follows:

Classification of steroids

(1) *Relatively non-polar.* Progesterone, androst-4-ene-3:17-dione, testosterone, and deoxycorticosterone; and the acetates of the last, 11-deoxy-17-hydroxycorticosterone, 11-dehydrocorticosterone, and corticosterone.

(2) *Relatively polar.* The known active cortical steroids (for review, see Reichstein & Shoppee, 1943), and the acetates of 11-dehydro-17-hydroxycorticosterone and 17-hydroxycorticosterone.

Table 2. *R_F values of various steroids obtained with partition systems using aqueous methanol as stationary phase*

(The values given are taken from 44 cm. flows on Whatman no. 4 paper at 34°. With higher temperatures the values increase. Errors of more than ±0·05 are rare, and are usually due to faulty equilibration. Mixtures of compounds III and IV, or IV and V, can only be separated completely by over-running with system *A* or *B*(3). Systems; see text (p. 372) for details. The 'amorphous fractions' are those of Wintersteiner & Pfiffner, 1936.)

	Partition systems						
Compounds	(*A*)	(B_1)	(B_2)	(B_3)	(B_4)	(B_5)	(*C*)
17-Hydroxycorticosterone (I)	0·0	0·03	0·10	0·01	0·15	0·32	0·4
11-Dehydro-17-hydroxycorticosterone (II)	0·01	0·05	0·20	0·02	0·30	0·50	0·7
Corticosterone (III)	—	0·23	0·65	0·15	0·70	0·85	—
11-Deoxy-17-hydroxycorticosterone (IV)	—	0·27	0·70	0·20	0·72	0·87	—
11-Dehydrocorticosterone (V)	—	0·36	0·75	0·25	—	0·96	—
11-Deoxycorticosterone (VI)	0·39	0·86	0·95	0·75	—	1·0	—
21-Acetate of I	—	0·25	—	—	—	—	—
21-Acetate of II	0·04	0·39	—	—	—	—	—
21-Acetate of IV	0·25	—	—	—	—	—	—
21-Acetate of VI	0·70	—	—	—	—	—	—
Testosterone	0·40	—	—	—	—	—	—
Androst-4-ene-3:11:17-trione	0·26	—	—	—	—	—	—
Androst-4-ene-3:17-dione	0·70	—	—	—	—	—	—
Progesterone	0·85	—	—	—	—	—	—
'Amorphous fraction' 1	0·0	—	—	—	—	0·25	0·3
'Amorphous fraction' 2	0·0	—	—	—	—	0·12	—
'Amorphous fraction' 3	0·0	—	—	—	—	0·03	—

(3) *Very polar.* The inactive polyhydroxy cortical steroids, such as *allo*pregnane-3β:11β:17β:20:21-pentol, and *allo*pregnane-3β:11β:17β:21-tetrol-20-one (Reichstein & Shoppee, 1943), and the unidentified components of the 'amorphous fraction' of the adrenal cortex (Wintersteiner & Pfiffner, 1936).

These three groups are conveniently handled by solvent systems *A*, *B* and *C*, respectively, where the aim is to examine an extract in a single run of 2–3 hr. If more time is available, better separations can be achieved by over-running with a system in which the steroids under study move with low R_F values.

Steroids less polar than progesterone would probably require a reversed phase system such as recently described by Kritchevsky & Calvin (1950).

Table 2 gives some R_F values obtained with the above type of steroid.

The steroids so far studied conform well to the postulate of Martin (1950) that, to a first approximation, in any given phase pair the partition coefficient of any compound in a series will be an additive function of its substituent groups. The properties and number of polar groups on an unknown steroid can be gauged fairly well by comparing their chromatographic behaviour with that of reference compounds in the above systems. The presence of acetylatable hydroxyl groups can be detected by the great increase in R_F value after acetylation, both on alumina paper and in partition systems.

Using temperatures of 15–25° equilibration is very slow (2–3 days) and with low concentrations of methanol streaky chromatograms result. The great improvement gained by using elevated temperatures and high concentrations of methanol is remarkable and probably related to the high molecular weight of the steroids combined with their low solubilities at room temperature and their strong adsorption on unwashed filter paper (Bush, 1950*a*), and on filter paper exhaustively washed by the method of Hanes & Isherwood (1949).

Concentration of extracts and spotting technique

This is always tedious with steroids, particularly since it is usually necessary to use the whole of a biological sample on one chromatogram. The problem is commonly to get all the steroid and a minimum of non-steroid material from *x* ml. of blood or urine on to 0·5 sq.cm. of filter paper. It is difficult to reduce any such extract to less than 1 ml. without running into serious volumetric errors when using volatile and mobile solvents, and the normal pipetting technique for paper chromatograms is very tedious with more than 20–40 μl. of solvent. Further, repetitive spotting with a pipette piles up the solutes as a ring on the paper, often dense enough to hinder dissolution and spoil a chromatogram.

The following techniques have therefore been devised to solve this problem.

Spotting with volumes of 40–100 μl.

The starting line is drawn well away from one end of the paper and marked, not with the usual spots, but with lanes, 5–10 mm. wide, running back from it at right angles (Fig. 1*a*). The solutions to be chromatographed are then pipetted

along, and within, their respective lanes and the solvent allowed to dry (Fig. 1*b*). If possible this is done in one operation so that an even deposition of solute occurs.

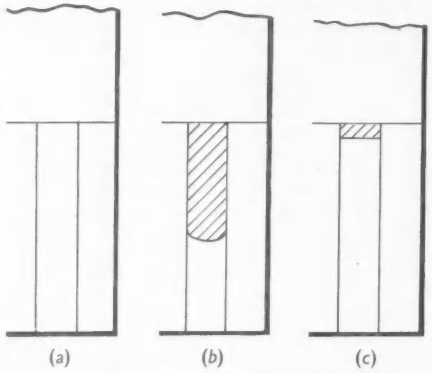


Fig. 1. Concentration of 0·02–0·2 ml. of extract for chromatography by a preliminary chromatogram (see text, p. 373).

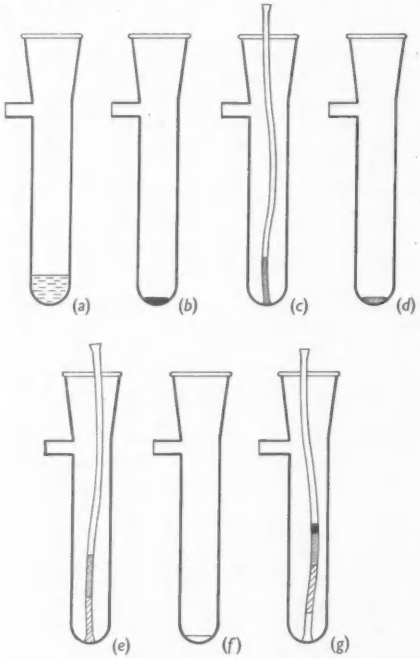


Fig. 2. Concentration of an extract for total deposition on a chromatogram (see text, p. 374).

The sheet is then hung in a small tank specially kept for the purpose with the lower edge dipping into a mixture of $CHCl_3$-ethyl acetate (1:1, v/v) containing a small addition of methanol. An ascending chromatogram is thus run with a solvent in which all the steroids move with the front, which is allowed to ascend to the starting line. The result, when dried, is that all the steroids are compressed into small rectangles on the starting line and are evenly deposited (Fig. 1*c*). This preliminary chromatogram takes 5–10 min.

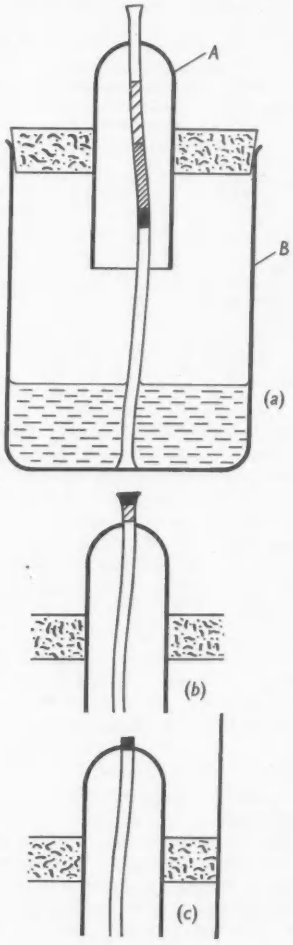


Fig. 3. Concentration of an extract for total deposition; concentration by a 'wick chromatogram' (see text, p. 375).

Total deposition of extracts (1–2 *ml.*)

The extract is made light pink by adding a minimal amount of Sudan Red and then evaporated *in vacuo* in a test tube with side arm to a small red drop at the bottom of the tube (Fig. 2*a*, *b*). This drop is taken up on to a three to five strand lamp wick, previously cleaned by 8 hr. Soxhlet

extraction with 90% ethanol (Fig. 2*c*). The tube is rinsed down with about 0·1 ml. methanol-ethyl acetate (1:1, v/v) and taken up on the wick. At the third wash the drop will be colourless (Fig. 2*f*) indicating complete recovery of the dye and lipoid solutes in the original extract.

The wick is then suspended in a glass cowl (Fig. 3*a*, *A*) as shown, with 1–2 cm. emerging. The beaker (*B*) is filled with a little ethyl acetate and this is allowed to rise up the wick. In about 15 min. the dye is concentrated on the emerging zone (Fig. 3*b*). Immediately before preparing the chromatogram the wick is drawn down till only 2–3 mm. emerge (Fig. 3*c*). When concentrated on this small zone, as indicated by the dye, it is simply painted on to a lane and treated as described above.

These methods have saved much time in the analysis of blood samples for steroids.

Extraction of steroids from blood samples

The following methods have been used for extracting cortical steroids from blood.

(*A*) The blood was stirred gradually into 4 vol. absolute methanol. After 15 min. the protein was filtered off at the pump with Whatman no. 1 paper, and the precipitate washed twice with 15 ml. methanol warmed to 45°. The filtrate was distilled to about 1 ml. *in vacuo* at 45°, cleared with minimal ethanol and added to 50 ml. acetone. After adding 2–3 drops saturated $MgCl_2$ in ethanol the acetone was chilled for 30 min. and then filtered through Whatman no. 1 at the pump. The precipitate was washed twice with 10 ml. cold acetone and then distilled (as above) to near dryness. The residue was taken up in 50 ml. of light petroleum (b.p. 40–60°) and the flask washed with 50 ml. 60% methanol. This methanol was used in equal fractions to extract the petroleum solution three times. The methanol extracts were combined and washed once with 15 ml. petroleum before distilling down to about 10 ml. aqueous solution. This was made up to 50 ml. 0·1 N-Na_2CO_3 with water and 0·5 N-Na_2CO_3 chilled in ice. The alkaline solution was immediately extracted with three portions of 15 ml. ethyl acetate. The combined ethyl acetate extracts were washed once with 10 ml. 0·1 N-HCl and once with 10 ml. water. The washings were back-extracted with 4 ml. ethyl acetate, and the combined ethyl acetate fractions dried over anhydrous Na_2SO_4.

(*B*) The blood was diluted with 1 vol. of water and shaken up well with 6 vol. of $CHCl_3$. The resulting emulsion was then cleared by chilling in ice and adding an excess of well powdered anhydrous Na_2SO_4. The clear $CHCl_3$ solution was filtered and distilled to 2 ml. at 45° *in vacuo* and added to 30 ml. acetone, saturated $MgCl_2$ added, and placed in ice for 0·5 hr. After filtration the acetone solution was distilled (as above) down to a gum which was taken up in 70% ethanol (30 ml.) and washed three times with 10 ml. light petroleum. The ethanol solution was then distilled to near dryness and taken up in minimal methanol, to which ten times its volume of ethyl acetate was then added. After 30 min. in ice the solution was centrifuged and the supernatant drawn off. The precipitate was then redissolved in methanol and reprecipitated by ethyl acetate.

The final extracts were then concentrated for chromatography by one of the methods described earlier (p. 373).

These methods are based on the work of Pfiffner & Vars (1934), Pfiffner, Wintersteiner & Vars (1935) and Grollman (1939). Recoveries have not been quantitatively checked, but chromatographic examination of all the rejected fractions in the above schemes showed no detectable cortical steroid. Using samples yielding 30–40 μg. of corticosterone or 17–hydroxycorticosterone in the final extract, and with a limit of detection at 5–7 μg. per chromatogram spot, this indicated that losses by co-precipitation or partition were less than 10–20%. Losses by destruction remain unknown, but there has been no indication that they are large.

Both methods yield final extracts which are clean enough for immediate partition chromatography when using the whole extract of up to 40 ml. blood. The first method is the more thorough and was generally used.

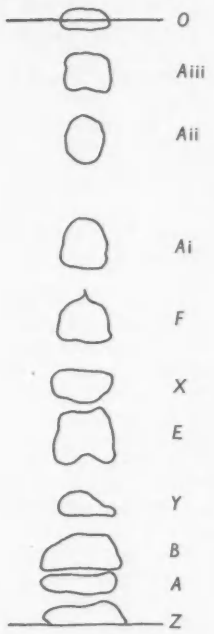


Fig. 4. Whole cortical extract run with system *B*5 on Whatman no. 4 at 34°. Photograph of the fluorescence developed by spraying with methanolic NaOH. *O*, starting line; *A* i, *A* ii, *A* iii, 'amorphous fraction' components; *A*, 11-dehydrocorticosterone; *B*, corticosterone; *E*, 11-dehydro-17-hydroxycorticosterone; *F*, 17-hydroxycorticosterone; *X*, unidentified blue fluorescence; *Y*, *Z*, unidentified spots; *Z*, solvent front.

RESULTS

In all the work so far only rough estimates of quantity have been made by comparing the density and area of spots (photographic) with spots containing known amounts of a reference compound. The spots have usually contained 5–20 μg.

Extracts of adrenal gland

The main object of study has been a semi-processed extract of 900 lb. (410 kg.) ox adrenals from Allen and Hanbury's Ltd. Results so far indicate the presence of appreciable amounts of all the three substances absorbing strongly in the ultraviolet, reacting with 2:4-dinitrophenylhydrazine and 2-hydroxy-3-naphthoic acid hydrazide (Camber, 1949), and falling in group 3 have been found on chromatograms of the 'amorphous fraction', with R_F values identical with similar substances from the above glandular extract (*A* i, *A* ii and *A* iii, in order of increasing polarity).

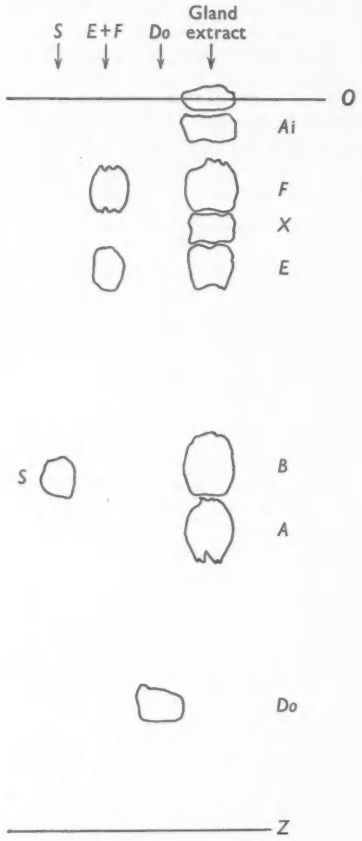


Fig. 5. Cortical extract and reference compounds run with system *B* 4. *A*, *B*, *E*, *F*, *O*, *X*, as in Fig. 4; *S*, 11-deoxy-17-hydroxycorticosterone; *Do*, deoxycorticosterone; *Z*, solvent front.

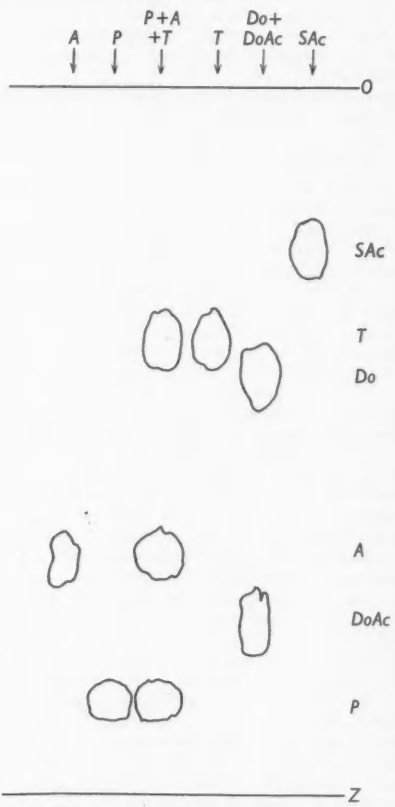


Fig. 6. Pure steroids of group I (see text) run with system *A*. *P*, progesterone; *T*, testosterone; *A*, androst-4-ene-3:17-dione; *Do*, deoxycorticosterone; *DoAc*, deoxycorticosterone acetate; *SAc*, 11-deoxy-17-hydroxycorticosterone acetate; *O*, starting line; *Z*, solvent front.

active cortical steroids previously described (Reichstein & Shoppee, 1943), except that deoxycorticosterone and 11-deoxy-17-hydroxycorticosterone have not yet been detected (cf. Zaffaroni, Burton & Keutmann, 1950). A large number of substances belonging to group 3 were found on chromatograms. By the great kindness of Dr O. Wintersteiner a sample of the original 'amorphous fraction' (Wintersteiner & Pfiffner, 1936) was available for comparison with these unknown substances. So far substance *A* i has been obtained substantially pure from such chromatograms and was assayed by Dr M. Vogt (Edinburgh) using the cold-exposure test of Selye & Schenker (1938), in which it was found to be very active. The sample was free of all known active cortical steroids but contained a trace of a fluorescent impurity.

These experiments are being further pursued.

Table 3. *Protocol of a typical experiment on the adrenal effluent of a dog*

(Dog, male (10·1 kg.) Nembutal anaesthesia. Continuous collection of adrenal venous blood from left gland. Blood pressure maintained by saline and 'Dextran' transfusion into femoral vein. Adrenal blood flow 2–5 ml./min. Each half-hour collection extracted and purified; one-tenth of each final extract used for chromatogram.)

Successive 30 min. collections	1	2	3	4	5
17-Hydroxycorticosterone on chromatogram (μg.)	8	18	16	18	18
Average secretion rate during each collection in mg./hr.	0·16	0·36	0·32	0·36	0·36

Extracts of adrenal venous blood

The presence of large amounts of 17-hydroxycorticosterone in adrenal venous blood has been demonstrated by isolation (Nelson, Reich & Samuels, 1950; Nelson, Reich & Zaffaroni, 1950) and by chromatography. Using the above methods, and the criteria of (1) chromatographic behaviour of the acetate with three different alumina-paper systems and three different partition systems; (2) ultraviolet absorption and fluorescence with sodium hydroxide; (3) ketone reaction with 2:4-dinitrophenylhydrazine and 2-hydroxy-3-naphthoic acid hydrazide; (4) iodine colour reaction on alumina paper, all being identical with pure 17-hydroxycorticosterone, as a satisfactory demonstration of this compound, it has been found as the chief steroid in adrenal venous blood in eleven dogs. The operative procedure was the same as that of Vogt (1943) except that nembutal was more commonly used as anaesthetic.

In addition the two least polar components of the 'amorphous fraction' (*A* i and *A* ii) seem to occur in the adrenal effluent though in smaller quantity. The identity with *A* i and *A* ii is not yet completely proven.

In two dogs appreciable amounts of a compound fulfilling the above criteria of identity with corticosterone appeared in the adrenal effluent in one-quarter to two-thirds of the amount of 17-hydroxycorticosterone during different periods of collection.

The amounts found in the adrenal effluent were of the same order as those arrived at by Vogt (1943) using the Selye-Schenker assay (see Table 3).

DISCUSSION

The methods described above were evolved largely with cortical steroids in mind, but the partition systems can handle any steroid as polar as, or more polar than, progesterone. The alumina-paper method can deal with steroids less polar than progesterone but its disadvantages outweigh its advantages. However, it is extremely useful with oestrogens; oestrone and oestradiol can be separated (and oestriol left on the origin) in a 10–15 min. ascending run and detected in amounts of 0·5 μg. by the iodine reaction.

The applications that have been made so far are only semi-quantitative, and quantitative estimations would probably be better done on columns as described by Butt *et al.* (1949). The paper method, however, lends itself readily to the biologist or biochemist who often needs qualitative or semi-quantitative results on a large number of different samples.

The results so far obtained by studying the dog adrenal venous blood are interesting when compared with the numerous biological assays that have been made on this material. Hartmann, Brownell & Thatcher (1947) described a 'fat factor' in adrenal extracts, with no glycogenic or electrolyte activity, which Hartmann & Liu (1950) found in the adrenal venous blood of dogs. Eight out of fourteen animals secreted appreciable amounts, but the rate of secretion was not affected by adrenocorticotropic hormone (ACTH) injections even when the glycogenic assay of the blood (Brownell, 1950) was greatly increased. Spencer (1950) found the equivalent of 4 μg./ml. deoxycorticosterone acetate in the serum from dog adrenal venous blood, using a bioassay based on acute salt retention in mice.

Nelson and his co-workers (Nelson, Reich & Samuels, 1950; Nelson, Reich & Zaffaroni, 1950) and Hechter (1950*a*, *b*) have shown that 17-hydroxycorticosterone is the predominant secretory product of the adrenals of dogs and oxen, but it is not clear whether the secretion of this one compound can explain all the complex physiological actions of the adrenal cortex. The regulation of sodium chloride excretion is a particularly difficult problem. Certainly Spencer's (1950) results could not be due to deoxycorticosterone itself, for a concentration of 1 μg./ml. serum would have been detectable on the chromatogram of the blood samples usually taken, and in one case where a much larger sample of blood was used the concentration of deoxycorticosterone must have been less than 0·2 μg./ml. to escape detection (cf. however, Hechter, 1950*a*, *b*).

While the above experiments throw no light on the functions of the various secretory products, they do show that the dog adrenal can secrete large amounts of 17-hydroxycorticosterone and corticosterone, and also small amounts of two compounds probably contained in the 'amorphous fraction' of cortical extracts. It does not, therefore, seem likely that adrenal function can be explained entirely in terms of the secretion of 17-hydroxycorticosterone, and its stimulation by ACTH.

SUMMARY

1. The use of alumina paper for the chromatography of steroids is described. The elimination of displacement effects due to impurities demands tedious purification of most biological extracts.

2. Partition systems of the aqueous methanol/hydrocarbon type can be run very conveniently on paper if certain special procedures are adopted. Extracts sufficiently pure to be handled by these systems can be simply and rapidly prepared.

3. Special methods for quick concentration of these extracts and their even deposition on filter paper are described.

4. Some applications to pure substances, extracts of adrenal glands, and adrenal venous blood are described.

5. A preliminary examination of the 'amorphous fraction' obtained from adrenal extracts by Wintersteiner & Pfiffner (1936) indicates the presence of at least three very polar substances with strong ultraviolet absorption near 254 mμ., two of which appear in adrenal venous blood.

The author would like to thank Mr J. Perkins and Mr D. N. Henty for the design and electrical fitting of the thermostatically controlled box used in these experiments, and the assistance of Mr K. Mourin at the operations.

He is also greatly indebted to Dr W. Feldberg and Dr R. K. Callow for much helpful advice and criticism; and to Prof. T. Reichstein for samples of pure steroids.

REFERENCES

Brante, G. (1949). *Nature, Lond.*, **163**, 651.
Brownell, K. A. (1950). *Fed. Proc.* **9**, 19.
Burton, R. B., Zaffaroni, A. & Keutmann, E. H. (1950). *Science*, **111**, 6.
Bush, I. E. (1950*a*). *Nature, Lond.*, **166**, 445.
Bush, I. E. (1950*b*). *J. Physiol.* **112**, 10*P*.
Butt, W. R., Morris, P. & Morris, C. J. O. R. (1949). *1st Int. Congr. Bioch. Abstr.* p. 405.
Camber, B. (1949). *Nature, Lond.*, **163**, 285.
Cornish, R. E., Archibald, R. C., Murphy, E. A. & Evans, H. M. (1934). *Industr. Engng Chem.* **26**, 397.
Datta, S. P., Overell, B. G. & Stack-Dunne, M. (1949). *Nature, Lond.*, **164**, 673.
Grollman, A. (1939). *J. Pharmacol.* **67**, 257.
Hanes, C. S. & Isherwood, F. A. (1949). *Nature, Lond.*, **164**, 1107.
Hartmann, F. A., Brownell, K. A. & Thatcher, J. S. (1947). *Endocrinology*, **40**, 450.
Hartmann, F. A. & Liu, T. Y. (1950). *Fed. Proc.* **9**, 57.
Hechter, O. (1950*a*). *Fed. Proc.* **8**, 70.
Hechter, O. (1950*b*). *Fed. Proc.* **9**, 58.
Kritchevshy, D. & Calvin, M. (1950). *J. Amer. chem. Soc.* **72**, 4330.
Martin, A. J. P. (1950). *Ann. Rev. Biochem.* **19**, 517.
Nelson, D. H., Reich, H. & Samuels, L. T. (1950). *Science*, **111**, 578.
Nelson, D. H., Reich, H. & Zaffaroni, A. (1950). *J. biol. Chem.* **187**, 411.
Pfiffner, J. J. & Vars, H. M. (1934). *J. biol. Chem.* **106**, 645.
Pfiffner, J. J., Vars, H. M. & Taylor, A. R. (1934). *J. biol. Chem.* **106**, 625.
Pfiffner, J. J., Wintersteiner, O. & Vars, H. M. (1935). *J. biol. Chem.* **111**, 585.
Reichstein, T. & Shoppee, C. W. (1943). *Vitamins & Hormones*, **1**, 345.
Reichstein, T. & Shoppee, C. W. (1949). *Discuss. Faraday Soc.* no. 7, 305.
Selye, H. & Schenker, V. (1938). *Proc. Soc. exp. Biol., N.Y.*, **39**, 518.
Spencer, A. G. (1950). *Nature, Lond.*, **166**, 32.
Vogt, M. (1943). *J. Physiol.* **102**, 341.
Wintersteiner, O. & Pfiffner, J. J. (1936). *J. biol. Chem.* **116**, 291.
Zaffaroni, A., Burton, R. B. & Keutmann, E. H. (1949). *J. biol. Chem.* **177**, 109.
Zaffaroni, A., Burton, R. B. & Keutmann, E. H. (1950). *Fed. Proc.* **9**, 250.

Kinetics of 'Acid' Phosphatase Action

By F. SCHØNHEYDER
Department of Biochemistry, Aarhus University, Denmark

(*Received* 31 *November* 1950)

Prostate 'acid' phosphatase is present in normal human seminal fluid in very high concentration (Kutscher & Wolbergs 1935). The optimum pH for acid phosphatase in sperm was found to be 4–6·2 depending upon the experimental conditions (Kutscher & Wörner, 1936; Lundquist, 1947). Lundquist computed the dissociation constant of the enzyme-substrate complex (Michaelis constant) from Kutscher & Wörner's experiments with β-glycerophosphate and on the basis of his own experiments with choline phosphate, phenyl phosphate and β-glycerophosphate.

Little attention has so far been paid to the reaction kinetics of the hydrolysis of phosphoric acid esters by acid phosphatase. In the present study reaction mechanisms are described which lead to simple

equations corresponding closely to the complete course of the hydrolysis of phenyl phosphate under varying experimental conditions.

EXPERIMENTAL

Enzyme. Solutions of unpurified acid prostate phosphatase from human sperm plasma in 2% NaCl were used. 1 ml. sperm plasma solution (about 3%) was added to 400 ml. substrate solution.

Substrate. Phenylphosphoric acid (m.p. 100°) dissolved in 2% NaCl was used. 0·01 g.mol. phenylphosphoric acid + 13 ml. N-NaOH were made up to 1 l. with 2% NaCl. Varying amounts of this stock solution were diluted with 2% NaCl to 400 ml.

Previous experience with a continuous titration technique in kinetic studies on the activity of pancreatic lipase (Schønheyder & Volqvartz, 1950) suggested the use of the same technique to follow the hydrolysis of phenyl phosphate at 22° and pH 5·62±0·05. pH is registered by means of a glass electrode and a Radiometer potentiometer. On addition of enzyme to the substrate solution at pH about 5·60 a progressive increase in pH is observed, which is counteracted by addition of acid at the rate required to keep the pH constant. The acid (HCl) was added through a capillary tube connected with a Geissler burette (divisions 0·05 ml.). The pH was measured immediately after the addition of enzyme and followed during the whole experiment, an adequate amount of acid being added after each rise in pH above 5·67. For each addition of acid is obtained a series of corresponding values of pH and time. pH is plotted as ordinate with time in minutes as abscissa, and the time corresponding to pH 5·62 is determined; $t=0$ corresponds to the addition of enzyme. If the pH is then below 5·62 a certain time will elapse before this pH is reached. During this period some of the substrate is hydrolysed, and in order to obtain figures proportional to the acid bound it is necessary to add a correction to all readings on the burette. Careful determination of this correction is of greatest importance to the mathematical treatment of the experimental results. The pH is displaced in an alkaline direction because phenylphosphoric acid is stronger than phosphoric acid. Walker & King (1950) found pK_2 5·73 for phenylphosphoric acid. The writer's investigations confirm this value and also show that pK_1 is about 1. It was found that after complete cleavage of 1 mmol. phenyl phosphate 0·395 m-equiv. of HCl was needed to bring the pH back to 5·62. This value agrees fairly well with the amount calculated from the pK values of phenylphosphoric acid and phosphoric acid. At pH 5·62 the amounts of substrate present and of phosphate produced are large enough to keep the pH within the limits of 5·62±0·05 by addition of HCl. On the other hand, the buffer capacity allows easy registration of changes in pH. Further addition of buffer substance to the enzyme-substrate mixture has therefore been omitted.

RESULTS

Optimum pH

The continuous titration technique is not suitable for the determination of the optimum pH, which was therefore determined by colorimetric measurement of the phenol liberated from phenyl phosphate. It appears from the pH-activity curve (Fig. 1) that the pH used for the continuous titration experiments (5·62) was close to the optimum. The author is indebted to Dr P. Strøier Rasmussen for this unpublished experiment.

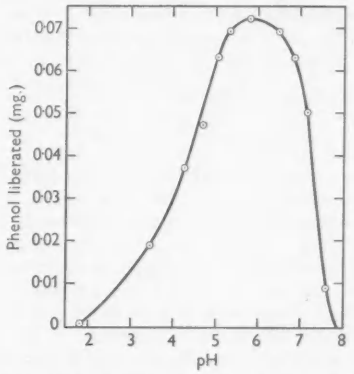


Fig. 1. Effect of pH on the activity of sperm acid phosphatase. Reaction mixture 5·9 ml. 0·01 M-phenyl phosphate in various buffers. The incubation was carried out at 37° for 15 min. The phenol released was determined by means of an adaptation of the King & Armstrong (1934) method for alkaline phosphatase.

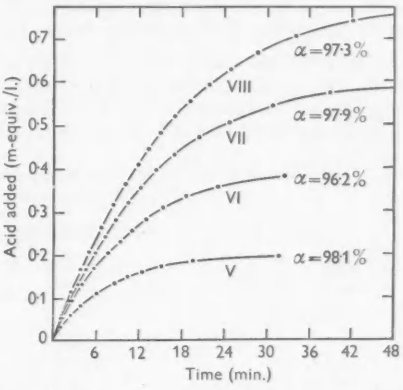


Fig. 2. Effect of varying phenyl phosphate concentration upon the rate of hydrolysis by acid phosphatase. α = highest degree of hydrolysis measured. V, 0·499 mM; VI, 0·998 mM; VII, 1·496 mM; VIII, 1·995 mM.

Effects of varying enzyme and substrate concentrations

Fig. 2 shows experiments performed by the continuous titration technique, using the same amount of enzyme with four different concentrations of phenyl phosphate. The hydrolysis was followed nearly to completion, which is important if the

kinetic constants are to be determined with accuracy.

The progress of the enzymic hydrolysis does not follow either zero-order or first-order kinetics. However, for given concentrations of enzyme and substrate, the experimental results are fitted with great accuracy by the rate equation:

$$-\frac{ds}{dt}=\frac{as}{b+cs}, \qquad (1)$$

whose integrated form is

$$at=b\ln\frac{s_o}{s}+c\,(s_o-s) \qquad (2)$$

(where s is the concentration of substrate at time t, s_o the concentration at zero time, and a, b, c are empirical constants). The value of a depends only on the enzyme concentration, b is independent of enzyme concentration but is a linear function of the initial substrate concentration, s_o; while c is a true constant, whose value is independent of both enzyme and substrate concentration.

Writing $A=b/a$ and $B=c/a$, eqn. 2 becomes

$$t=A\ln\frac{s_o}{s}+B\,(s_o-s). \qquad (3)$$

For any given enzyme concentration, the values of A and B remain constant throughout the course of hydrolysis. Their values were determined by the usual method for the graphical solution of simultaneous equations. First $A=t/\ln\frac{s_o}{s}$ for $B=0$, and $B=t/(s_o-s)$ for $A=0$ were computed for all observations. On a graph with abscissa A and ordinate B, straight lines were drawn through the points $(B, 0)$ and $(0, A)$ for each value of t. If the observations fit eqn. 3, all the lines will meet in a point whose abcissa and ordinate are the required values of A and B. This method is far simpler than the 'method of least squares'.

In Table 1, the values of A and B determined in this way have been used to calculate by eqn. 3 the time, $t_{calc.}$, corresponding to each observed value of s.

Table 1. *Hydrolysis of* 2·494 *mM-phenyl phosphate, corresponding to* 0·9838 *mM-HCl. Exp. XII*

$t_{obs.}$ (min.)	HCl added ($mM_{cor.}$)	$t_{calc.}$ $\left(13{\cdot}5\ln\frac{s_o}{s}+5{\cdot}6\,(s_o-s)\right)$
0·47	0·0252	0·49
1·20	0·0631	1·25
2·35	0·1135	2·29
3·33	0·1639	3·38
4·57	0·2144	4·52
5·70	0·2648	5·72
6·90	0·3153	6·98
8·26	0·3657	8·32
9·70	0·4161	9·76
10·80	0·4540	10·90
12·03	0·4918	12·11
13·33	0·5296	13·40
14·88	0·5675	14·79
16·20	0·6053	16·29
17·85	0·6431	17·92
19·79	0·6809	19·73
21·59	0·7188	21·74
24·05	0·7566	24·03
26·78	0·7944	26·71
29·50	0·8323	29·93
32·33	0·8575	32·53
35·35	0·8827	35·69
39·40	0·9079	39·71
45·00	0·9331	45·32
54·90	0·9584	54·83
69·00	0·9710	64·24

$\alpha=98{\cdot}7\,\%$

Table 2. *Effect of substrate concentration (s_o) and addition of phosphate (p_o) on the rate of hydrolysis of phenyl phosphate*

(Same amount of enzyme used for each experiment.)

Exp. no.	s_o (mM)	p_o (mM)	B	$A_{obs.}$	$A_{calc.}$	$\frac{A_{calc.}\ln\frac{s_o}{s}+\bar{B}(s_o-s)}{t}$	$v_o=\frac{s_o}{A+Bs_o}$
V	0·499	—	5·7	7·6	7·6	0·999	0·0478
VI	0·998	—	6·0	9·45	9·5	1·003	0·0646
VII	1·496	—	5·8	11·5	11·5	0·998	0·0741
VIII	1·995	—	5·6	13·5	13·4	0·995	0·0809
IX	2·494	—	5·8	15·4	15·4	0·997	0·0835
X	2·494	1·247	5·6	20·0	20·2	1·011	0·0734
XI	2·494	2·494	6·2	25·2	25·1	0·993	0·0613
	Average		**5·8**			**0·999**	

Table 3. *Effect of enzyme concentration on the rate of hydrolysis of phenyl phosphate*

Exp. no.	s_o (mM)	Enzyme	B	A	v_o	E	EB	EA
XII	2·494	Undil.	5·6	13·5	0·0908	1·087	6·1	14·7
XIII	2·494	50 %	11·6	26·4	0·0468	0·560	6·5	14·8
XIV	2·494	25 %	20·9	53	0·0234	0·280	5·9	14·8
						Average	**6·1**	**14·8**

The results show close agreement with the observed times, $t_{obs.}$.

Table 2 (Exps. V–IX) shows that with a constant amount of enzyme, the value of A increases linearly with the initial substrate concentration s_o, while the value of B remains constant. Table 3 gives the results of three experiments with the same substrate concentration and different amounts of enzyme. In agreement with eqns. 2 and 3, the observed values of A and B are inversely proportional to the enzyme

where k is the velocity constant for the break-up of the enzyme-substrate complex and K_m is the Michaelis constant. The integrated form of (5) is

$$t=\frac{K_m}{kE}\ln\frac{s_o}{s}+\frac{1}{kE}(s_o-s) \qquad (6)$$

(cf. Henri, 1903), which is formally similar to eqn. 3. The similarity is only apparent, however, since the value of A in eqn. 3 varies with the substrate concentration, while K_m/kE does not.

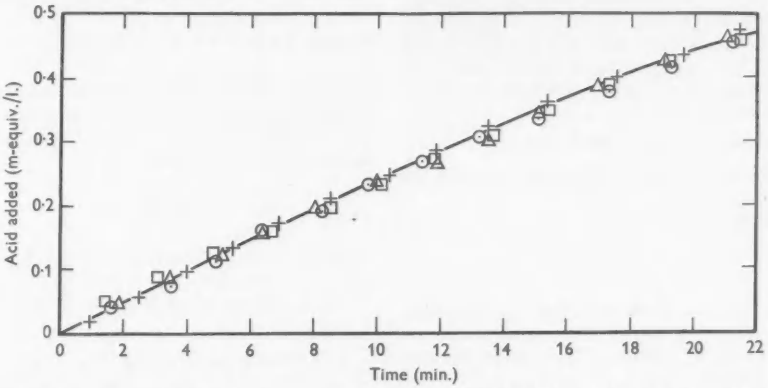


Fig. 3. Effect of varying phenol concentration upon the rate of hydrolysis of phenyl phosphate by acid phosphatase. I (⊙), 2·494 mM-phenyl phosphate; II (□), 2·494 mM-phenyl phosphate + 1·247 mM-phenol; III (△), 2·494 mM-phenyl phosphate + 2·494 mM-phenol; IV (+), 2·494 mM-phenyl phosphate + 4·988 mM-phenol.

concentration E; hence the products EA and EB are constants in the three experiments. (Since the pure enzyme is not available, the true value of E is unknown; in these calculations the value $E=1$ has been arbitrarily assumed for the enzyme concentration used in Exp. IX.)

If eqn. 1 is written as $-\frac{ds}{dt}=\frac{s}{A+Bs}$, then the initial velocity v_o is given by

$$v_o=\left(-\frac{ds}{dt}\right)_{t=0}=\frac{s_o}{A+Bs_o}. \qquad (4)$$

The values of v_o calculated in this way are given in Tables 2 and 3. With constant enzyme concentration, v_o increases with s_o, approaching a limiting value when $Bs_o \gg A$ (Table 2). With constant substrate concentration v_o is directly proportional to the enzyme concentration (Table 3).

Effects of phenol and orthophosphate

The above results show that the kinetics of acid phosphatase are more complicated than would be expected from the theory of Michaelis & Menten (1913). This theory predicts the rate equation

$$-\frac{ds}{dt}=\frac{kEs}{K_m+s}, \qquad (5)$$

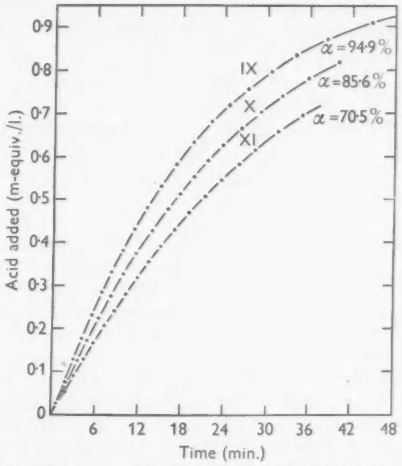


Fig. 4. Effect of varying phosphate concentration upon the rate of hydrolysis of phenyl phosphate by acid phosphatase equal to the amount used in experiments portrayed in Fig. 2. α = highest degree of hydrolysis measured. IX, 2·494 mM-phenyl phosphate; X, 2·494 mM-phenyl phosphate + 1·247 mM-phosphate; XI, 2·494 mM-phenyl phosphate + 2·494 mM-phosphate.

An explanation of the difference could be that the activity of acid phosphatase is inhibited by the products of the hydrolysis of phenyl phosphate, i.e. free phenol and orthophosphate. Fig. 3 shows that phenol, in concentrations up to 5 mM, has no effect on the kinetics. Fig. 4 shows that orthophosphate, on the other hand, is definitely inhibitory.

Table 2 (Exps. IX–XI) shows that with constant enzyme and substrate concentration, the addition of orthophosphate decreases the initial velocity v_o. Also, the value of the constant A increases linearly with the initial concentration of orthophosphate, p_o, while the value of constant B is unaffected. The data from all the experiments of Table 2, both with and without added phosphate, are found to fit the linear regression equation

$$A_{\text{calc.}} = 5{\cdot}63 + 3{\cdot}90\ (s_o + p_o), \tag{7}$$

as is shown by the good agreement between the values of $A_{\text{obs.}}$ and $A_{\text{calc.}}$ in Table 2.

It follows from eqn. 3 that

$$\frac{1}{t}\left[A \ln \frac{s_o}{s} + B(s_o - s)\right] = 1.$$

The values of this expression, calculated from $A_{\text{calc.}}$ and $\bar{B}$ (the mean value of B in all experiments), are given in Table 2, column 7, and all lie between 0·993 and 1·011.

THEORETICAL

The following reaction scheme leads to equations analogous to eqns. 1–3, and is in good agreement with the experimental findings:

$$e + s \underset{k_2}{\overset{k_1}{\rightleftharpoons}} (es), \tag{8}$$

$$(es) + \mathrm{H_2O} \xrightarrow{k_3} e + p + f, \tag{9}$$

$$e + p \underset{k_5}{\overset{k_4}{\rightleftharpoons}} (ep) \tag{10}$$

(where s = substrate, p = phosphate, f = phenol, e = free enzyme, (es) = enzyme-substrate complex, (ep) = enzyme-phosphate complex, and the k's are velocity constants).

The equations for the formation of the enzyme-substrate complex (8) and its break-up into free enzyme and products (9) are identical with those of Briggs & Haldane (1925); but in addition the free enzyme is supposed to combine reversibly with phosphate to form an inactive complex (10).

From eqns. 8–10 we can at once write down the differential equations:

$$-\frac{ds}{dt} = k_1 es - k_2(es), \tag{11}$$

$$\frac{dp}{dt} = k_3(es) + k_5(ep) - k_4 ep, \tag{12}$$

$$\frac{df}{dt} = k_3(es). \tag{13}$$

In the steady state, $-ds/dt = dp/dt = df/dt$; hence the right-hand sides of these three equations may be equated.

Hence from eqns. 11 and 13,

$$k_1 es - k_2(es) = k_3(es),$$

or

$$\frac{e \times s}{(es)} = \frac{k_2 + k_3}{k_1} = K_s, \tag{14}$$

where K_s is the Michaelis constant or apparent dissociation constant of the enzyme-substrate complex (cf. Briggs & Haldane, 1925). Similarly, from eqns. 12 and 13 we obtain

$$\frac{e \times p}{(ep)} = \frac{k_5}{k_4} = K_p, \tag{15}$$

where K_p is the dissociation constant of the enzyme-phosphate complex.

Let E be the total enzyme concentration (free and bound). Then

$$E = e + (es) + (ep) = e\left[1 + \frac{s}{K_s} + \frac{p}{K_p}\right] \tag{16}$$

(from eqns. 14 and 15).

If p_o and s_o are the concentrations of p and s at zero time, then $p = p_o + s_o - s$; inserting this value of p into eqn. 16 and rearranging

$$e = E \Big/ \left[1 + \frac{(p_o + s_o)}{K_p} + \left(\frac{1}{K_s} - \frac{1}{K_p}\right) s\right]. \tag{17}$$

In the steady state $-\frac{ds}{dt} = k_3(es) = \frac{k_3}{K_s}\, es$.

Hence from (17)

$$-\frac{ds}{dt} = \frac{k_3 E s}{\left[K_s + (p_o + s_o)\frac{K_s}{K_p}\right] + \left[1 - \frac{K_s}{K_p}\right] s}, \tag{18}$$

which on integration gives

$$k_3 Et = \left[K_s + (p_o + s_o)\frac{K_s}{K_p}\right] \ln \frac{s_o}{s} + \left[1 - \frac{K_s}{K_p}\right](s_o - s). \tag{19}$$

This is analogous to eqns. 2 and 3 if we put

$$A = \frac{b}{a} = \frac{K_s}{k_3 E} + \frac{K_s(p_o + s_o)}{k_3 E}, \tag{20}$$

$$B = \frac{c}{a} = \frac{1}{k_3 E}\left[1 - \frac{K_s}{K_p}\right]. \tag{21}$$

Hence if E is constant, B should be constant, while A should increase linearly with $(p_o + s_o)$, which agrees with the experimental findings.

From eqn. 18 and the above expressions for A and B, it will be seen that when $p_o = 0$, the initial velocity is given by

$$v_o = \frac{k_3 E s_o}{K_s + s_o} = \frac{s_o}{A + B s_o}$$

identical with the Michaelis-Menten equation (5). Rearranging, this becomes

$$\frac{s_o}{v_o} = \frac{K_s}{k_3 E} + \frac{s_o}{k_3 E}. \qquad (22)$$

Hence a plot of s_o/v_o against s_o should give a straight line with slope $1/k_3E$, ordinate intercept K_s/k_3E, and abscissa intercept K_s. Fig. 5 shows the data of

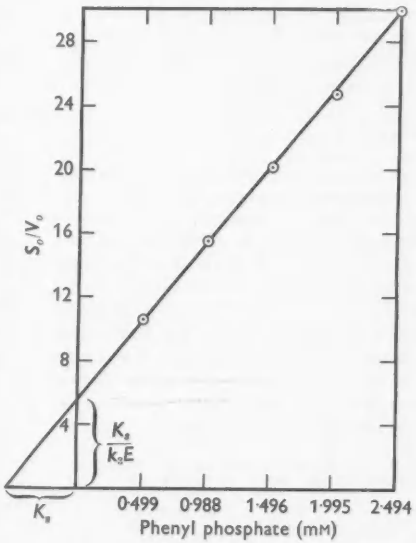


Fig. 5. Initial velocity-substrate concentration relationship (Exps. V–IX) plotted according to eqn. 22.

Exps. V–IX plotted in this way; the points lie very close to the straight line fitted by the 'method of least squares'. The value of the Michaelis constant, K_s, calculated from these data is 0·590 mM, and the value of k_3E is 0·104 mM/min. The true value of k_3 could only be determined if the enzyme concentration E were known in absolute units, which would only be possible if the pure enzyme were available.

From these values of K_s and k_3E and the experimentally determined constant B, the value of K_p can be determined by eqn. 21. Using the average value $\bar{B}$ obtained from all the experiments in Table 2, the average value of K_p comes to 1·49 mM. Thus the affinity of orthophosphate for enzyme is about 0·4 times that of phenylphosphate.

DISCUSSION

All the experimental results agree with the equations derived from the reaction scheme suggested, which postulates a reversible inhibition of the enzyme by orthophosphate. This does not necessarily prove that the scheme is correct. The somewhat different reaction scheme:

$$e + s \underset{h_2}{\overset{h_1}{\rightleftharpoons}} (ef) + p,$$

$$H_2O + (ef) \overset{h_3}{\rightharpoonup} e + f,$$

can be shown to give rise to equations identical with (2) and (3), the integrated form of the rate equation being

$$h_3 E t = \frac{1}{h_1}[h_3 + h_2(s_o + p_o)] \ln \frac{s_o}{s} + \left[1 - \frac{h_2}{h_1}\right](s_o - s). \qquad (23)$$

This means that the empirical constants, from which h_1, h_2 and h_3 may easily be calculated, will have a somewhat different significance.

Kinetic studies alone cannot decide which reaction mechanism is correct; the one given in the text is more in line with the mechanism usually postulated for hydrolytic enzymes. The problem may be solved when the pure enzyme is available; it should then be possible to decide whether the reversible combination with phosphate predicted by eqn. 10 actually occurs.

SUMMARY

1. By means of a continuous titration method adding dilute hydrochloric acid the kinetics of the enzymic decomposition of phenyl phosphate by unpurified acid prostate phosphatase at 22° has been studied. The effect of substrate concentration and excess of phenol and orthophosphate is reported. In all the experiments the hydrolysis was followed almost to completion. The pH optimum was determined by measuring the phenol liberated.

2. Two reaction mechanisms are put forward, both of which lead to equations that fit the experimental data with great accuracy over the whole course of the reaction.

REFERENCES

Briggs, G. E. & Haldane, J. B. S. (1925). *Biochem. J.* **19**, 338.
Henri, V. (1903). *Lois générales de l'action des diastases.* Paris: Thèse.
King, E. J. & Armstrong, A. R. (1934). *Canad. med. Ass. J.* **31**, 376.
Kutscher, W. & Wolbergs, H. (1935). *Hoppe-Seyl. Z.* **236**, 237.
Kutscher, W. & Wörner, A. (1936). *Hoppe-Seyl. Z.* **239**, 109.
Lundquist, F. (1947). *Acta physiol. scand.* **14**, 263.
Michaelis, L. & Menten, M. L. (1913). *Biochem. Z.* **49**, 333.
Schønheyder, F. & Volqvartz, K. (1950). *Biochim. Biophys. Acta*, **6**, 147.
Walker, P. G. & King, E. J. (1950). *Biochem. J.* **47**, 93.

The Enzymically Catalysed Transfer of the Deoxyribosyl Group from One Purine or Pyrimidine to Another

By W. S. MACNUTT* (Fellow of the Jane Coffin Childs Memorial Fund for Medical Research)
Institute of Cytophysiology and Biochemistry, University of Copenhagen, Denmark

(*Received* 30 *January* 1951)

Since several of the natural deoxyribosides serve equally well as growth-promoting substances for certain bacteria (Kitay, MacNutt & Snell, 1949, 1950; Hoff-Jørgensen, 1949; Kocher & Schindler, 1949) it seems probable that in such organisms the administered deoxyriboside is converted into the other deoxyribosides of which the bacterial deoxyribonucleic acid is constituted. The only mechanism which has been established for the biological synthesis of nucleosides (Kalckar, 1947; Friedkin & Kalckar, 1950) would depict the interconversion as follows:

$$\text{Deoxyribose-1-R} + \text{H}_3\text{PO}_4 \overset{\text{Enzyme}}{\rightleftharpoons} \text{Deoxyribose-1-phosphoric acid} + \text{R}$$

$$\text{Deoxyribose-1-phosphoric acid} + \text{R}^1 \overset{\text{Enzyme}}{\rightleftharpoons} \text{Deoxyribose-1-R}^1 + \text{H}_3\text{PO}_4$$

where R, R^1, etc. represent the purines and pyrimidines (or their precursors) which are characteristic of the deoxyribonucleic acid of the bacterium.

The experiments reported below were designed to test such a mechanism in representatives of the group of bacteria which require deoxyribosides for growth. Dialysed enzyme preparations from *Lactobacillus helveticus* S have been shown, by a series of indirect methods, to be capable of bringing about a wide variety of interconversions when incubated with a deoxyriboside and a free purine or pyrimidine. Contrary to expectation, deoxyribose-1-phosphate did not react, in this system, with purines or pyrimidines to yield deoxyribosides. These findings have been reported in brief (MacNutt, 1950). Enzyme solutions have been prepared from two additional bacteria, both of which require deoxyribosides for growth, and these, too, have been found to catalyse an exchange. The exchange of the deoxyribosyl group from one acceptor to another can hardly proceed through hydrolysis followed by resynthesis, since hydrolysed deoxyribose-1-phosphate was also inactive in this system. The enzyme preparation is therefore considered to be a trans-*N*-glycosidase, analogous to the trans-*O*-glycosidase from *Pseudomonas saccharophila* (Doudoroff, Barker & Hassid, 1947).

* Present address: University Chemical Laboratory, Cambridge.

EXPERIMENTAL

Materials

All melting points reported are uncorrected. Optical rotations were measured in a 2 dm. tube.

Purines and pyrimidines. Commercial preparations (Schwartz Laboratories; Hoffmann La Roche and Co.) were used. 5-Methylcytosine was generously supplied by Dr H. K. Mitchell. It melted at 276–278° with gas evolution after softening at 268°. (Found in material dried at 100° over P_2O_5 for 4 hr. (1 mm.): C, 48·1; H, 5·8; N, 33·7. Calc. for $C_5H_7ON_3$: C, 48·0; H, 5·6; N, 33·6%.) The picrate melted at 297–299° (decomp.).

4-Aminoglyoxaline-5-carbonamide hydrochloride was prepared from formamidomalonanamidine hydrochloride (a gift from Dr D. W. Woolley) by ring closure (Shaw & Woolley, 1949). Buff-coloured material was obtained which decomposed at 253° without melting completely. The picrate decomposed at 237°.

Deoxyribosides. Adenine deoxyriboside was kindly supplied by Dr T. G. Brady. The other deoxyribosides were prepared by a modification of Klein's (1938) procedure. Sodium deoxyribonucleate (25 g.) (7·55% P), prepared by Hammarsten's (1924) method, was dissolved in 500 ml. of water, adjusted to pH 8·0 with 15 N-NH_4OH, and 500 mg. of $MgSO_4.7H_2O$ in 5 ml. of water were added followed by 1 mg. of crystalline deoxyribonuclease (Nutritional Biochemicals Corporation) in 3 ml. of water. After a few minutes at 23° the viscosity of the solution had dropped. After 2 hr. 100 ml. of saturated $(NH_4)_2SO_4$ solution, 50 g. of $MgSO_4.7H_2O$ in 100 ml. of water, sufficient 15 N-NH_4OH to bring the pH to 8·7, and water to make a total volume of 800 ml. were added, followed by 200 ml. of calf intestinal phosphatase purified to the stage prior to reprecipitation with 0·8 saturated $(NH_4)_2SO_4$ (Schmidt & Thannhauser, 1943). The solution was covered with toluene and incubated at 37° for 2·5 days, NH_4OH being added from time to time to maintain the pH at 8·7–9·0.

Klein's (1938) procedure was followed for the separation of guanine deoxyriboside as a gel. The two portions of the gel were dissolved in 300 ml. of water and extracted with 250 ml. portions of *n*-butanol saturated with water until 500 ml. of washed butanol solution gave no appreciable amount of deoxyriboside upon evaporation. The total volume of *n*-butanol used was 4·5 l. The combined washed butanol layers were evaporated *in vacuo* to yield a white crystalline residue consisting of guanine deoxyriboside and hypoxanthine deoxyriboside from which 1·23 g. of guanine deoxyriboside were obtained by repeated crystallization from hot water. The compound began darkening around 208° (moderate rate of heating), continued to darken slowly as the temperature rose, and at 305° was dark brown. It did not shrink or decompose suddenly. $[\alpha]_D^{25°} = -42·7°$ in 1 N-NaOH (*c*, 2). The ultraviolet absorption is shown in Fig. 1. (Found in material dried in air at 25°: C, 41·3; H, 5·5; N, 24·6. Calc. for $C_{10}H_{13}O_4N_5.1\frac{1}{2}H_2O$: C, 41·3; H, 5·4; N, 24·2%.) (Found in material dried to constant weight over P_2O_5 at 100° (4 hr.; 1 mm.): C, 42·3; H, 5·0; N, 25·0%. Calc. for $C_{10}H_{13}O_4N_5.H_2O$: C, 42·1; H, 5·3; N, 24·6%.) (Found in material dried to constant weight over P_2O_5 at 140° (4 hr.; 1 mm.): C, 44·6; H, 5·2; N, 26·4, 26·3%. Calc. for $C_{10}H_{13}O_4N_5$: C, 44·9; H, 4·9; N, 26·2%.)

Crude hypoxanthine deoxyriboside was separated by Klein's (1938) procedure. To this was added the crude hypoxanthine deoxyriboside from the butanol extract. The yield of substance crystallized three times from hot water was 1·55 g. Further crystallization was required to obtain the pure compound. (Found in material dried in air at 25°; C, 47·2; H, 4·7; N, 22·4. Calc. for $C_{10}H_{12}O_4N_4$: C, 47·6; H, 4·8; N, 22·2%.) At about 206° the compound shrank somewhat in volume and began darkening slowly until at 310° it was brown. It did not melt or decompose suddenly. The ultraviolet absorption spectrum is shown in Fig. 2. $[\alpha]_D^{26°} = -21·5°$ in water (*c*, 1).

Thymine deoxyriboside (Klein's (1938) procedure). Yield of material melting at 182–184° = 0·53 g. The compound was crystallized once more from hot water: m.p. = 186–187°. (Found in material dried in air at 25°; C, 49·7; H, 5·7; N, 11·9. Calc. for $C_{10}H_{14}O_5N_2$: C, 49·6; H, 5·8; N, 11·6%.) $[\alpha]_D^{23°} = +18·5°$ in water (*c*, 2). The ultraviolet absorption spectrum is shown in Fig. 3.

Cytosine deoxyriboside was separated as the picrate by Klein's procedure (1938). The yield of the recrystallized salt was 2·37 g. It decomposed (black) without melting at 188–192°. (Found in material dried in air at 25°: C, 39·3; H, 3·2; N, 18·3. Calc. for $C_{15}H_{16}O_{11}N_6$: C, 39·5; H, 3·5; N, 18·4%.) Cytosine deoxyriboside was obtained in 80% yield by decomposition of the picrate. It crystallizes easily from hot absolute ethanol: m.p. 207–209°. (Found in material dried in air at 25°: C, 48·1; H, 5·8; N, 18·8. Calc. for $C_9H_{13}O_4N_3$: C, 47·6; H, 5·8; N, 18·5%.) $[\alpha]_D^{23°} = +57·6°$ in water (*c*, 2). The ultraviolet absorption spectrum is shown in Fig. 4.

Thymine deoxyriboside and cytosine deoxyriboside were more rapidly and conveniently prepared by the action of intestinal phosphatase upon the pyrimidine nucleotides. The dibarium salts (14·6 g.) of the pyrimidine diphosphonucleotides (Levene, 1921) were dissolved in cold water. $(NH_4)_2SO_4$ was added to precipitate the barium. The filtrate and washings were diluted to 500 ml.; 8 g. of $MgSO_4.7H_2O$ and 50 ml. of saturated $(NH_4)_2SO_4$ solution were added. The pH was adjusted to 9·0 with 15 N-NH_4OH; 50 ml. of calf intestinal phosphatase (Schmidt & Thannhauser, 1943) were added, and the solution was incubated as described previously. The filtrate was worked up for thymine deoxyriboside and cytosine deoxyriboside as before. Yield of thymine deoxyriboside of m.p. 184–185° = 0·44 g. Yield of cytosine deoxyriboside picrate (decomp. without melting at 184–189°) = 1·03 g.

Uracil deoxyriboside was prepared by the chemical deamination of cytosine deoxyriboside: 0·5 g. of cytosine deoxyriboside (m.p. 207–209°) was dissolved in 3·5 ml. of water. $NaNO_2$ (1·5 g.) and 1·5 ml. of glacial acetic acid were added. After 8·2 hr. at 24°, 0·4 g. of $NaNO_2$ and 0·8 ml. of glacial acetic acid were added. After standing at 24° for an additional 12 hr. the acetic acid was distilled off at 40° *in vacuo*. Water (30 ml.) was added, and the solution was again taken to dryness. The residue was dissolved in 180 ml. of water and 50 g. of moist H^+-charged amberlite IR-100 were added to the solution. To remove the acetic acid released, the filtrate and washings were reduced to 10 ml. (inside temperature 15°). Water (20 ml.) was added, and the solution was reduced as before to 4 ml. The pH was adjusted to 7 with $Ba(OH)_2$. Absolute ethanol (100 ml.) was added, whereupon a yellow precipitate formed and was discarded. The filtrate was reduced to dryness. The white crystalline mass was taken up in 6 ml. of hot absolute ethanol, and a small amount of insoluble material was filtered off. Upon reducing the filtrate to 4 ml. on a hot-water bath and placing the beaker at room temperature crystallization began (10 min.). The material was filtered off, sucked dry, and washed with a small portion of absolute ethanol. Yield: 0·285 g., m.p. 162·5–163°. Second crop: 0·038 g., m.p. 162–163°. 65% of the theoretical was recovered in the combined portions. The compound was crystallized twice more from absolute ethanol. It separated in clusters of platelets about 1 mm. long; m.p. 163–163·5°. It solidifies upon cooling and melts the second time at 161–162·5°. $[\alpha]_D^{26°} = +30·0°$ in water (*c*, 2). (Found in material dried in air at 25°: C, 47·7; H, 5·3; N, 12·2. Calc. for $C_9H_{12}O_5N_2$: C, 47·6; H, 5·3; N, 12·3%.) The spectral data (Fig. 5) were identical with those reported for uridine (Ploeser & Loring, 1949).

A crude barium salt of deoxyribose-1-phosphate was prepared by a method similar to that reported (Friedkin, 1950). Hypoxanthine deoxyriboside (400 mg.) yielded 200 mg. of crude barium deoxyribose phosphate. Found: *ortho* P, precipitated as $MgNH_4PO_4$, nil; *ortho* P, precipitated as $MgNH_4PO_4$, after hydrolysis for 10 hr. at pH 4·0 and 22° (determined by the method of Berenblum & Chain, 1938),

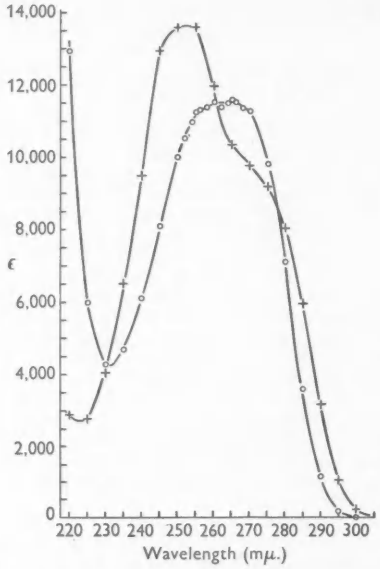


Fig. 1. Guanine deoxyriboside. *E* calc. on the air-dried sample as $C_{10}H_{13}O_4N_5.1\frac{1}{4}H_2O$.

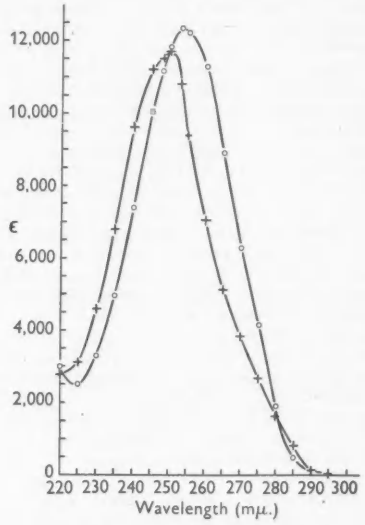


Fig. 2. Hypoxanthine deoxyriboside.

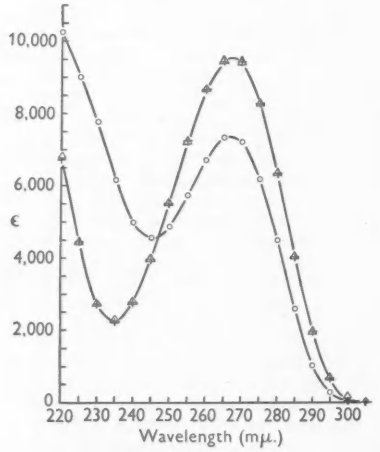


Fig. 3. Thymine deoxyriboside.

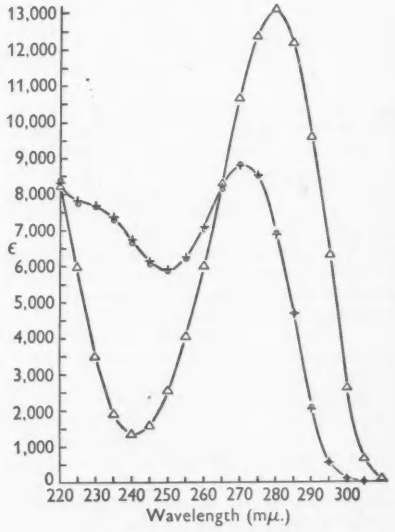


Fig. 4. Cytosine deoxyriboside.

Figs. 1–5. Ultraviolet absorption spectra of deoxyribosides. The solutions at concentrations of 10–12 μg./ml. were examined in a Beckman spectrophotometer. +—+, pH at 7·0 (weakly buffered with phosphate); ○—○, pH at about 12 (30 μl. of 5N-NaOH added per 3 ml. of weakly buffered solution); △—△, pH at about 1 (50 μl. of 15N-HCl added per 3·03 ml. of alkaline solution).

3·75%. Barium deoxyribose-1-phosphate content, calculated from the labile P, =43%. Yield=16% of the theoretical. 10 μl. (0·125 μmol.) of deoxyribose-1-phosphate (calc. upon the basis of the labile P), incubated with 125 μg. of hypoxanthine in 125 μl. of 0·1 M-glycine buffer (pH, 8·8) and 125 μl. (1·9 mg. dry wt.) of calf liver nucleoside phosphorylase (Friedkin & Kalckar, 1950) for 11 hr. at 24°, showed a net synthesis of 0·115 μmol. of deoxyriboside as judged by the microbiological test.

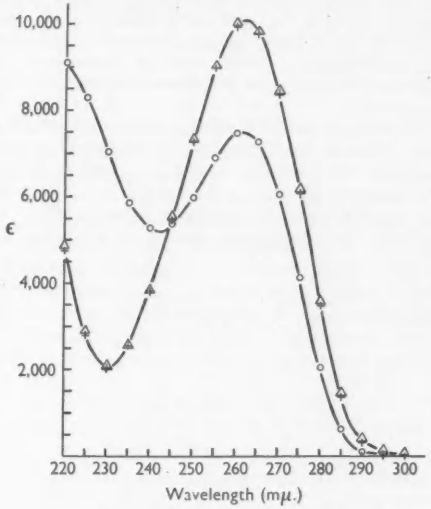


Fig. 5. Uracil deoxyriboside.

Bacterial enzymes. *Lactobacillus helveticus* S and *Lb. delbrueckii* ATCC 9649 were transferred to the inoculum medium and grown in 250 ml. lots of the complete basal medium as previously described (MacNutt & Snell, 1950). Thymidine was used as the growth-promoting substance at a level of 1 mg./250 ml. of final medium. After 24 hr. incubation at 37° the cells from 500 ml. of medium were collected by centrifugation and washed twice with 75 ml. portions of 0·05 M-citrate buffer at pH 6·0. The washed cells were suspended in 15 ml. of the same buffer and were broken up by agitation with glass beads for 30 min. in the electric tuning fork shaker (Mickle apparatus). During this process the cell suspension was cooled intermittently. Occasionally this suspension of broken bacteria was used, in which case it is referred to as 'undialysed enzyme'. 'Dialysed enzyme' was prepared by dialysing the above suspension against distilled water at 3°. The insoluble material was removed by centrifugation at low speed after the period of dialysis. The solution, preserved by freezing, retained its activity relatively well. The enzyme from *Thermobacterium acidophilus* R 26 was prepared as above from cells grown in a basal medium (Hoff-Jørgensen, 1951), supplemented with thymidine as described above.

Methods

The microbiological assay of deoxyribosides with *Thermobacterium acidophilus* R 26 was employed according to the described method (Hoff-Jørgensen, 1952). Thymine deoxyriboside was used as the standard.

Chromatography. One-dimensional ascending chromatography on paper was carried out in glass jars 11 × 48 cm. The compounds were placed along Whatman no. 4 filter paper in quantities of 10–20 μg. of purine or pyrimidine per cm. The solvent (25–30 ml.) was added to the dry vessel; the paper was suspended above the solvent, and the system equilibrated for the periods indicated. The distance traversed by the solvent was 45 cm. No special precautions were taken to ensure a constant temperature (usually about 23°), and R_F values varied somewhat on independent runs. The following systems were employed: *n*-butanol-water-ammonia: 1 ml. of 15 N-NH_4OH was added to 100 ml. of *n*-butanol saturated with water. After shaking thoroughly the clear supernatant was used. Equilibrated for 11 hr. Time to traverse 45 cm. = 1·5–2 days. *n*-Butanol-water-acetic acid: 25 ml. of *n*-butanol saturated with water + 5 ml. of glacial acetic acid.

Table 1. *R_F values of deoxyribosides and related compounds*

Compound	R_F values in system: *n*-Butanol-water-ammonia	*n*-Butanol-water-acetic acid	*n*-Butanol-water-ethyl acetate-morpholine	*n*-Butanol-water-morpholine-methyl glycol
Adenine	0·40	—	0·48	0·45
Guanine	0·15*	—	—	—
Hypoxanthine	0·19	—	0·36	—
Cytosine	0·28	—	—	—
Uracil	0·33	—	—	—
Thymine	0·50	—	—	—
4-Aminoglyoxaline-5-carbonamide	0·32	—	0·44	—
Adenine deoxyriboside	0·41	—	0·70	0·66
Guanine deoxyriboside	0·18	—	0·57	0·58
Hypoxanthine deoxyriboside	0·17	—	0·54	0·55
Cytosine deoxyriboside	0·26	0·35	0·70	0·68
Uracil deoxyriboside	0·34	0·44	0·88	0·77
Thymine deoxyriboside	0·48	0·56	0·94	0·85
Cytidine	0·15	—	—	—
5-Methylcytosine	0·36	—	—	—
Inosine	0·08	—	—	—
Uric acid	0·02	—	—	—

* It has been pointed out by Markham & Smith (1949) that the capacity of the system for guanine is very limited.

Equilibrated for 8 hr. Time to traverse 45 cm. = 1·5–2 days. *n*-Butanol-water-ethyl acetate-morpholine: 5 ml. of *n*-butanol saturated with water + 5 ml. of ethyl acetate saturated with water + 10 ml. of morpholine equilibrated for 24 hr. Time to traverse 45 cm. = 2–2·5 days. *n*-Butanol-water-morpholine-methylglycol: 10 ml. of *n*-butanol saturated with water + 10 ml. of morpholine + 5 ml. of methylglycol + 2 ml. of water. Equilibrated for 24 hr. Time to traverse 45 cm. = 3–3·5 days. The R_F values of the deoxyribosides and certain related compounds in the several systems are shown in Table 1.

RESULTS

The formation of thymine deoxyriboside by interaction of hypoxanthine deoxyriboside and thymine

The ease with which purine deoxyribosides are hydrolysed by acid (Levene & London, 1929) affords a convenient way of selectively destroying all the purine deoxyribosides in a mixture. This has also been demonstrated microbiologically (Hoff-Jørgensen, 1952). Thus, if a pyrimidine deoxyriboside were formed by the interaction of a purine deoxyriboside and a pyrimidine, it could easily be detected by the growth-promoting effect upon *Thermobact. acidophilus* R 26 after the purine deoxyriboside had been hydrolysed by dilute acid.

It was assumed at the outset that the exchange of the aglycone would proceed through the phosphorolytic cleavage of the nucleoside, followed by the exchange of the phosphate group of deoxyribose-1-phosphate for another purine or pyrimidine. For this reason xanthine oxidase was included in the incubation mixture in the following experiment.

Hypoxanthine deoxyriboside (0·78 μmol., 40 μl.), 1 ml. of 0·2 M-phosphate buffer at pH 8·04, 1 mg. of thymine, 1 ml. of undialysed *Lb. helveticus* enzyme (20 mg. dry wt.), and xanthine oxidase (1 mg. of protein, 50 μl.) from cream (Kalckar, Kjeldgaard & Klenow, 1950) were incubated at 37° (toluene added) for 10 hr. The control tube differed from the experimental in that hypoxanthine deoxyriboside was added after acidification. After adding 70 μl. of 5 N-HCl the tubes were heated in a boiling-water bath for 5 min.; water was added and the precipitate filtered off. The filtrate was neutralized with NaOH and assayed microbiologically. From the difference in the activity between control and experimental samples the total amount of pyrimidine deoxyriboside formed was computed.

To identify the pyrimidine deoxyriboside which had been formed, the solutions were freed from protein and excess inorganic salts and chromatographed on paper: eight-tenths of each sample (8 ml.) was reduced to 0·7 ml. at 70°. Absolute ethanol (5 ml.) was added. The supernatant and washings were evaporated to dryness at 65°, and the residue was extracted with 2 ml. of warm absolute ethanol. The filtrate and washings were again reduced to dryness. Water (1 ml.) was added to the residue. A sample (100 μl.) was placed along 4 cm. of paper and chromatographed in the *n*-butanol-water-ammonia system. Indicator spots: thymine, thymine deoxyriboside and uracil. Beneath ultraviolet light the compounds appeared as dark spots on the paper.

The thymine-thymine deoxyriboside spots ($R_F = 0·51$) were cut out and extracted with water. Upon the basis of the difference in microbiological activity between the filtrates of the control and experimental spots, the total amount of pyrimidine deoxyriboside recovered in the spot at $R_F = 0·51$ was calculated. The spots of hypoxanthine which appeared on the chromatogram were also extracted with water, the amounts present being estimated enzymically (ΔE_{290}) (Kalckar, 1947). Any hypoxanthine set free by the bacterial enzyme should be immediately oxidized to uric acid by the xanthine oxidase present. Hence, the difference in hypoxanthine content between control and experimental areas should serve as a measure of the hypoxanthine released from the nucleoside during the course of the reaction.

In this experiment 0·55 μmol. of pyrimidine deoxyriboside (71 % conversion) was formed in the reaction mixture and 0·36 μmol. (65 % of the pyrimidine deoxyriboside found) was recovered from the spot on the chromatogram corresponding to thymine deoxyriboside. The poor recovery by

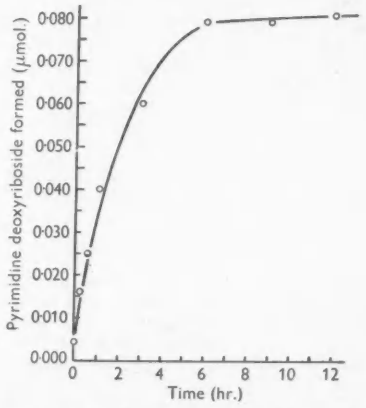


Fig. 6. The rate of formation of thymine deoxyriboside from hypoxanthine deoxyriboside plus thymine. 0·099 μmol. of hypoxanthine deoxyriboside, 125 μg. of thymine in 125 μl. of 0·2 M-phosphate buffer (pH 8·0), and *Lb. helveticus* enzyme (dialysed 7 hr.) 125 μl. (2·4 mg. dry wt.). The reaction (at 37°) was initiated by the addition of enzyme and was stopped by the addition of 7 μl. of 5 N-HCl. The samples were hydrolysed and assayed as usual.

chromatography was most likely due to losses in manipulation, since only 0·49 μmol. hypoxanthine (63 % of the original) was accounted for on the control chromatogram. These results suggest strongly that thymine exchanged enzymically with the hypoxanthine of hypoxanthine deoxyriboside to give thymine deoxyriboside. The amount of hypoxanthine released from the nucleosidic linkage (0·47 μmol.) agreed fairly well with the amount of thymine deoxyriboside formed (0·36 μmol.).

It was subsequently found that the exchange occurred equally well in the absence of phosphate buffer and xanthine oxidase. This led to a testing of deoxyribose-1-phosphate and a source of 2-deoxy-D-ribose (hydrolysed deoxyribose-1-phosphate) in the exchange system. Both proved to be ineffective. These results are reported below.

The rate of the reaction. In Fig. 6 the rate of formation of thymine deoxyriboside from hypoxanthine deoxyriboside plus thymine is presented. Under the conditions specified, equilibrium had been established within 6 hr. An incubation period of 10–11 hr. was adopted for all the subsequent experiments. It will be noted that in all the experiments a considerable excess of the purine or pyrimidine was employed.

The formation of various pyrimidine deoxyribosides

Various pyrimidines were tested for their ability to react with hypoxanthine deoxyriboside to give pyrimidine deoxyribosides active as growth factors for *Thermobact. acidophilus* R 26. Thymine, uracil, and 5-methylcytosine were active (Table 2). Since

Table 2. *The ability of various pyrimidines to form microbiologically active pyrimidine deoxyribosides by the exchange reaction*

(0·39 μmol. of hypoxanthine deoxyriboside, 500 μg. of pyrimidine in 500 μl. of 0·05 M-alanylglycine buffer (pH 8·5), and 500 μl. (1·7 mg. dry wt.) of *Lb. helveticus* enzyme (dialysed 4 days). Hydrolysed and assayed as usual.)

Pyrimidine tested	Pyrimidine deoxyriboside formed (μmol.)
Thymine	0·18
Uracil	0·17
5-Methylcytosine	0·20*
Methyl thiouracil	<0·005
5:5-Diethylbarbituric acid	<0·005
4:5:6-Triaminopyrimidine	<0·005

* Carried out in phosphate buffer (pH 8·04).

the exchange proceeded in the absence of phosphate there was no evidence that nucleoside phosphorylase played any important function in the transfer of the deoxyribosyl group from hypoxanthine to the pyrimidine in question.

Uracil deoxyriboside from hypoxanthine deoxyriboside. Uracil was incubated with hypoxanthine deoxyriboside as in the experiment with thymine except that dialysed (3 days) *Lb. helveticus* enzyme (4 mg. dry wt.) was used. The preparation of the solution for chromatography also differed in that the residue was extracted four times with boiling absolute ethanol (1·5 ml. each time). Chromatographic separation was carried out in the *n*-butanol-water-acetic acid system. Indicator spots: thymine deoxyriboside, uracil deoxyriboside, and cytosine deoxyriboside.

Hydrolysis and assay showed that 0·76 μmol. of pyrimidine deoxyriboside was formed from 0·78 μmol. of hypoxanthine deoxyriboside (97% conversion), while chromatography (Table 3) showed that 90% of the pyrimidine deoxyriboside formed was recovered from the spot with $R_F = 0{\cdot}40$ in the *n*-butanol-water-acetic acid system—the same value as that of uracil deoxyriboside. Uracil deoxyriboside, showed an activity of 1·03 per μmol. in the microbiological assay, where 1 μmol. of thymine deoxyriboside = 1·00. Uracil deoxyriboside is thus as active as the other natural deoxyribosides in supporting the growth of *Thermobact. acidophilus* R 26.

Thymine deoxyriboside and uracil deoxyriboside from guanine deoxyriboside. Guanine deoxyriboside (0·75 μmol.) was incubated with thymine as described in Table 3, the only difference being that xanthine oxidase was omitted. Hydrolysis and microbiological assay revealed that 0·56 μmol. of pyrimidine deoxyriboside had been formed (75% conversion). Chromatography of the ethanolic extract of the hydrolysed solutions in the *n*-butanol-water-ammonia system and assay of the appropriate areas showed that 0·48 μmol. (86%) of the pyrimidine deoxyriboside formed was accounted for in the thymine deoxyriboside spot ($R_F = 0{\cdot}49$). The uracil deoxyriboside spot ($R_F = 0{\cdot}32$) and the cytosine deoxyriboside spot ($R_F = 0{\cdot}25$) each showed less than 0·01 μmol. of pyrimidine deoxyriboside.

Similar figures were obtained when uracil was used. 0·54 μmol. (72% conversion) of pyrimidine deoxyriboside was formed, of which, by chromatography in the *n*-butanol-water-acetic acid system, 0·47 μmol. (87%) was accounted for in the uracil deoxyriboside spot ($R_F = 0{\cdot}39$). The thymine deoxyriboside ($R_F = 0{\cdot}49$) and cytosine deoxyriboside ($R_F = 0{\cdot}30$) spots each showed less than 0·01 μmol. of pyrimidine deoxyriboside.

Cytosine deoxyriboside from hypoxanthine deoxyriboside and cytidine. 0·20 μmol. of hypoxanthine deoxyriboside, 400 μg. of cytidine (Hoffman-La Roche), 200 μl. of 0·2 M-phosphate buffer at pH 8·04, and 200 μl. of *Lb. helveticus* enzyme (dialysed 7 hr.; 3·9 mg. dry wt.) were incubated as usual. The reaction was stopped by the addition of 7 ml. of ethanol. In the control tube the hypoxanthine deoxyriboside was added after ethanol had been added. The ethanolic extract was prepared and chromatographed in the *n*-

Table 3. *Formation of uracil deoxyriboside from hypoxanthine deoxyriboside*

(0·76 μmol. total pyrimidine deoxyriboside was found by direct assay of the hydrolysed mixture.)

	Deoxyriboside found (μmol.)		
Sample	Thymine deoxyriboside spot ($R_F = 0{\cdot}50$)	Uracil deoxyriboside spot ($R_F = 0{\cdot}40$)	Cytosine deoxyriboside spot ($R_F = 0{\cdot}32$)
Control	<0·01	<0·01	<0·01
Experimental	0·02	0·70	0·01

butanol-water-ammonia system. Found: 0·13 μmol. of cytosine deoxyriboside (R_F = 0·26) formed, and 0·12 μmol. of hypoxanthine deoxyriboside (R_F = 0·14) disappeared.

Since large amounts of cytosine appeared on the chromatogram it seems likely that the reaction involved the prior splitting of cytidine to give cytosine which then entered into the exchange reaction. The difference in the rate at which these enzyme preparations split the ribosides and the deoxyribosides is remarkable (see Kalckar, MacNutt & Hoff-Jørgensen, 1952). It should be noted that cytosine deoxyriboside was not deaminated by the enzyme preparation.

Thymine deoxyriboside and uracil deoxyriboside from cytosine deoxyriboside. 0·44 μmol. of cytosine deoxyriboside (40 μl.), 500 μl. of pyrimidine, 500 μl. of *Lb. helveticus* enzyme (dialysed 3 days, 4·0 mg.), 500 μl. of 0·2 M-phosphate buffer at pH 8·04 were incubated and hydrolysed as usual. The ethanolic extract was chromatographed in the *n*-butanol-water-acetic acid system. The results (Table 4) show that thymine and uracil exchange enzymically with the cytosine of cytosine deoxyriboside. Since the residual cytosine deoxyriboside from the exchange reaction with thymine (Exp. A) was not deaminated to uracil deoxyriboside the origin of uracil deoxyriboside in Exp. B must proceed by the exchange of the entire pyrimidine or by a transamination involving positions six of cytosine deoxyriboside and uracil. This latter possibility was not excluded, but an analogous case in the purine series (adenine and hypoxanthine deoxyriboside) has been shown to involve the transfer of the purine skeleton (Kalckar *et al.* 1952).

5-Methylcytosine deoxyriboside and its chemical deamination to give thymine deoxyriboside. 0·37 μmol. of guanine deoxyriboside, 500 μl. of 5-methylcytosine, 500 μl. of 0·2 M-phosphate buffer at pH 8·04, and 500 μl. of *Lb. helveticus* enzyme (dialysed 1 day, 1·7 mg.) were incubated. Ethanol was added and the ethanolic extract was chromatographed in the *n*-butanol-water-ammonia system. The spots were assayed as usual. The results (Table 5) indicated that 5-methylcytosine deoxyriboside had an R_F value of 0·32, which would distinguish it from thymine deoxyriboside and cytosine deoxyriboside but not from uracil deoxyriboside (R_F = 0·30). The presumed 5-methylcytosine deoxyriboside was next separated from uracil deoxyriboside in the *n*-butanol-water-morpholine-methyl glycol system. The results were as follows: thymine deoxyriboside formed (R_F = 0·83), <0·01 μmol.; uracil deoxyriboside formed (R_F = 0·75), <0·01 μmol.; deoxyriboside formed (R_F = 0·66), 0·25 μmol.; guanine deoxyriboside lost (R_F = 0·57), 0·22 μmol. The presumed 5-methylcytosine deoxyriboside has an R_F value in the *n*-butanol-water-morpholine-methyl glycol system which lies close to that of cytosine deoxyriboside.

Thus, by means of chromatography in two systems, it was shown that 5-methylcytosine reacted enzymically with purine deoxyribosides to yield a pyrimidine deoxyriboside which differed from thymine, uracil and cytosine deoxyribosides. To identify it as 5-methylcytosine deoxyriboside the substance was converted into thymine deoxyriboside by treatment with HNO_2.

Portions (50 μl.) of the ethanolic extracts were placed on two sheets and chromatographed in the *n*-butanol-water-ammonia system. From one of them the desired areas were removed and saved for assay. From the other, the control and experimental areas corresponding to the location of the new deoxyriboside (R_F = 0·35) were excised and extracted with boiling ethanol. The supernatant and washings were evaporated to dryness on the water bath. The residues were treated with 65 μl. of water, 65 μl. of glacial acetic acid, and 70 mg. of $NaNO_2$ added in portions over a period of 2 days. The acetic acid was distilled off, the residues were dissolved in water and treated with 2 g. of H^+-charged amberlite IR-100-H. The filtrate and washings were reduced to 2 ml. at 40°. $Ba(OH)_2$ was added to adjust the pH to 8·0. CO_2 was passed in to neutralize the excess of $Ba(OH)_2$. The residues were extracted with ethanol, but since considerable amounts of inorganic material dissolved, the final extraction was carried out with ethyl acetate. The residues were transferred to the paper and chromatographed in the *n*-butanol-water-ammonia system. The appropriate areas were cut out and assayed microbiologically. Since, of the five deoxyribosides tested (Kitay *et al.* 1949) *Leuconostoc citrovorum* 8081 responds only to thymine deoxyriboside, the untreated and the deaminated substances were tested as possible growth factors for this organism. The results (Table 6) show that the substance presumed to be 5-methylcytosine deoxyriboside was inactive as a growth factor for *Leuc. citrovorum* and was converted into thymine deoxyriboside by treatment with HNO_2. The recovery was poor (15–16%).

Table 4. *Conversion of cytosine deoxyriboside into thymine and uracil deoxyriboside*

			Deoxyriboside found (μmol.)		
Exp.	Pyrimidine added	Sample	Thymine deoxyriboside spot (R_F = 0·54)	Uracil deoxyriboside spot (R_F = 0·44)	Cytosine deoxyriboside spot (R_F = 0·35)
A	Thymine	Control	<0·01	<0·01	0·33
		Experimental	0·32	<0·01	0·04
B	Uracil	Control	<0·01	0·01	0·36
		Experimental	<0·01	0·41	0·05

Table 5. *Formation of 5-methylcytosine deoxyriboside from guanine deoxyriboside*

	Deoxyriboside found (μmol.)			
Sample	Thymine deoxyriboside spot (R_F = 0·46)	5-Methylcytosine deoxyriboside spot (R_F = 0·32)	Cytosine deoxyriboside spot (R_F = 0·25)	Guanine deoxyriboside spot (R_F = 0·16)
Control	<0·01	0·01	<0·01	0·30
Experimental	<0·01	0·23	<0·02	0·11

Table 6. *Deamination of 5-methylcytosine deoxyriboside to thymine deoxyriboside*

Treatment	Net amount* of deoxyriboside found (μmol. $\times 10^{-5}$)			
	Assayed with *Thermobact. acidophilus* R 26		Assayed with *Leuc. citrovorum* 8081†	
	Thymine deoxyriboside spot (R_F = 0·47)	5-Methylcytosine deoxyriboside spot (R_F = 0·34)	Thymine deoxyriboside spot (R_F = 0·48)	5-Methylcytosine deoxyriboside spot (R_F = 0·34)
None	8	850	1	2
Deaminated with nitrous acid	140	9	130	−1

* The amount in the experimental sample minus the amount in the control sample (thymine deoxyriboside as the standard).

† 48 hr. growth in a described medium (MacNutt & Snell, 1950).

The formation of purine deoxyribosides

When purines are tested in the exchange system by the reverse process to that shown in Table 2, the exchange of any purine with the pyrimidine of a pyrimidine deoxyriboside will cause a loss in the growth-promoting activity of the mixture after hydrolysis, whether the purine deoxyriboside formed be itself active or inactive as a growth factor for *Thermobact. acidophilus*. Also, were the pyrimidine deoxyriboside merely split by the enzyme every purine tested would seem to enter into the exchange reaction, unless the purine were by chance an inhibitior of the hydrolysis. Since previous experiments had shown that relatively good yields of pyrimidine deoxyribosides could be obtained it did not seem likely that the splitting of the pyrimidine deoxyriboside would offer serious difficulty. Results showing the amount of deoxyriboside split by this enzyme preparation in the absence of an acceptor (about 20 %) are given later.

Table 7. *Ability of various purines and related compounds to enter into the exchange reaction*

(0·368 μmol. of thymine deoxyriboside, 500 μg. of the given compound in 500 μl. of alanyl-glycine buffer, etc. as in Table 2.)

Purine or related compound	Purine deoxyribose formed (μmol.)
Adenine	0·260
Guanine	0·155*
Hypoxanthine	0·295
Xanthine	0·300
4-Aminoglyoxaline-5-carbonamide.HCl	0·295
	0·303†
Formamido malonamidine.HCl	0·02
Uric acid	0·02
2:6-Diaminopurine.$\frac{1}{2}H_2O$.$\frac{1}{2}H_2SO_4$	0·03
4:5:6-Triaminopyrimidine	0·03
Formyl-4:5:6-triaminopyrimidine	−0·01
Dihydrothymine‡	0·02†

* Since guanine is so insoluble its concentration could not be brought to that of the other compounds tested.

† Phosphate buffer at pH 8·04.

† 0·44 μmol. of cytosine deoxyriboside incubated with 500 μg. of dihydrothymine (a gift from Dr H. K. Mitchell).

Four purines and 4-aminoglyoxaline-5-carbonamide (Table 7) apparently reacted enzymically with thymine deoxyriboside to give purine deoxyribosides, in that there was a loss of growth-promoting activity after mild treatment of the mixture with acid. Dihydrothymine was tested as though it were a purine, since it has been shown (Levene & Bass, 1931) that dihydrouridine is as easily hydrolysed by acid as purine ribosides. The negative result with uric acid was further investigated to determine whether it was due to failure to enter into the exchange reaction or to the formation of a microbiologically active deoxyriboside which resists mild acid hydrolysis, since uric acid riboside shows abnormal resistance to acid hydrolysis (Davis, Newton & Benedict, 1922).

Thymine deoxyriboside, uric acid, phosphate buffer, and enzyme were incubated as usual and treated with ethanol; the ethanolic extract was chromatographed in the *n*-butanol-water-ammonia system. In this system one would expect uric acid deoxyriboside to have an R_F value close to that of uric acid (R_F = 0·02) and certainly much lower than that of thymine deoxyriboside. The amount of thymine deoxyriboside (R_F = 0·48) recovered from the experimental sample (0·45 μmol.) was in very good agreement with that of the control (0·46 μmol.). It is inferred, therefore, that uric acid did not enter into the exchange reaction.

The formation of adenine deoxyriboside from uracil deoxyriboside. A further investigation of the purine deoxyriboside formed by the interaction of adenine and pyrimidine deoxyribosides showed that adenine deoxyriboside was formed and that it was not deaminated to hypoxanthine deoxyriboside.

Uracil deoxyriboside (0·44 μmol.), adenine (500 μg.), etc. were incubated in the usual way. The ethanolic extract of the unhydrolysed incubation mixture was chromatographed in the *n*-butanol-water-ammonia system, and the chromatogram assayed. 0·37 μmol. of adenine deoxyriboside (R_F = 0·40) was formed, 0·45 μmol. of uracil deoxyriboside (R_F = 0·34) disappeared, and less than 0·01 μmol. of hypoxanthine deoxyriboside (R_F = 0·15) was formed.

The exchange of one purine for another. Since the enzyme preparation was without adenine deoxyriboside deaminase activity, the exchange of adenine for the hypoxanthine of hypoxanthine deoxyriboside and the guanine of guanine deoxyriboside could easily be demonstrated (Table 8). The possibility that transamination between positions six of adenine and hypoxanthine deoxyriboside occurs has been ruled out by use of radioactive adenine (Kalckar *et al.* 1951).

The unknown deoxyriboside formed from the reaction of xanthine with uracil deoxyriboside. Uracil deoxyriboside (0·44 μmol.), xanthine (500 μg.), etc., were incubated, the

Table 8. *Exchange of adenine for the hypoxanthine of hypoxanthine deoxyriboside and the guanine of guanine deoxyriboside*

(Purine deoxyriboside, 500 μg. of adenine, and conditions as in Table 5.)

		Deoxyriboside found (μmol.)		
Deoxyriboside added	Sample	Adenine deoxyriboside spot (R_F = 0·40–0·42)	Hypoxanthine deoxyriboside and guanine deoxyriboside spots (R_F = 0·16–0·15)	All other areas
Hypoxanthine deoxyriboside (0·41 μmol.)	Control	<0·01	0·32	<0·01
	Experimental	0·12	0·14	<0·01
Guanine deoxyriboside (0·40 μmol.)	Control	<0·01	0·38	<0·01
	Experimental	0·28	0·05	<0·01

Table 9. *The deoxyriboside formed when 4-aminoglyoxaline-5-carbonamide is incubated with thymine deoxyriboside*

(Thymine deoxyriboside (0·44 μmol.), 4-aminoglyoxaline-5-carbonamide.HCl (500 μg.) in 500 μl. of 0·2 M-phosphate buffer (pH 8·04), 500 μl. (1·7 mg.) of *Lb. helveticus* enzyme, dialysed 1 day, were incubated as usual. The total 'purine deoxyriboside' formed, estimated by hydrolysis and assay was 0·37 μmol.)

	Deoxyriboside found by chromatography of the unhydrolysed samples in the *n*-butanol-water-ammonia system and assay of the appropriate areas (μmol.)			
Sample	Thymine deoxyriboside spot. R_F = 0·49	Adenine deoxyriboside. R_F = 0·39	Hypoxanthine and guanine deoxyribosides. R_F = 0·16	Unknown deoxyriboside spot. R_F = 0·31
Control	0·47	0·01	0·01	0·03
Experimental	0·10	0·01	0·02	0·10

ethanolic extract prepared and chromatographed as in Table 5. Beneath ultraviolet light the control sample showed two spots: R_F = 0·05 (xanthine) and R_F = 3·04 (uracil deoxyriboside). In the experimental sample three spots appeared: R_F = 0·34 (uracil and residual uracil deoxyriboside), R_F = 0·05 (xanthine), and a third dark area, partially overlapping the xanthine spot, at R_F = 0·06. The areas at R_F = 0·34, R_F = 0·05–0·06, and R_F = 0·18–0·20 (where hypoxanthine deoxyriboside and guanine deoxyriboside would appear) were assayed and gave the following results: Uracil deoxyriboside lost, 0·37 μmol.; unknown deoxyriboside (R_F = 0·05–0·06) formed, 0·16 μmol.; hypoxanthine or guanine deoxyriboside formed, 0·02 μmol. Since in the *n*-butanol-water-ammonia system the known deoxyribosides and their respective purines and pyrimidines have R_F values which are close together one would expect xanthine deoxyriboside to appear near the xanthine spot. The R_F value of the unknown deoxyriboside was less than that of any of the known deoxyribosides; whether it was actually xanthine deoxyriboside was not further investigated.

The quantitative aspects of this experiment were very unsatisfactory, only 43% of the uracil deoxyriboside which disappeared being recovered in the form of the unknown deoxyriboside. There was a pronounced drift in the microbiological assay of this substance, which possibly means that the shape of the growth curve with the unknown deoxyriboside differed from that of the standard curve with thymine deoxyriboside. The substance may not have been as active in supporting growth as an equimolar quantity of thymine deoxyriboside; greater losses than those encountered in the case of other deoxyribosides may have occurred in the preparation of solutions of this substance for chromatography; or actual loss may have occurred when this substance was autoclaved with the medium (pH 6·8) at 120°.

The reaction product of 4-aminoglyoxaline-5-carbonamide and thymine deoxyriboside. It may be seen from Table 8 that a 'purine deoxyriboside' was apparently formed when 4-aminoglyoxaline-5-carbonamide was incubated with thymidine in the presence of the enzyme from *Lb. helveticus*. Since it has been claimed that 4-amino-5-imidazole carboxamide is a precursor of purines in bacteria (Shive, Ackermann, Gordon, Getzendaner & Eakin, 1947) the possibility that the deoxyriboside of this compound had been synthesized enzymically was further examined.

A typical balance experiment (Table 9) shows that the amount of 'purine deoxyriboside' formed (0·37 μmol.), as judged by the loss of microbiological activity following hydrolysis, agreed well with the amount of thymine deoxyriboside which disappeared (0·37 μmol.), as judged by chromatography of the unhydrolysed mixtures and assay. But the amount of unknown deoxyriboside formed (0·07 μmol.), as measured by direct assay of the chromatogram, was only 19% of the thymine deoxyriboside which disappeared, an even lower yield of purine deoxyriboside than that obtained from the reaction of xanthine and uracil deoxyriboside (43%). In the microbiological assay of this substance, too, the drift was serious. The data merely suggested that a purine deoxyriboside, which differed from all of the known purine deoxyribosides in having an R_F value of 0·31 in the *n*-butanol-water-ammonia system and which showed some microbiological activity toward *Thermobact. acidophilus* R 26, was formed in the reaction between

4-aminoglyoxaline-5-carbonamide and thymine deoxyriboside.

Another experiment was therefore carried out to identify the product more certainly. Thymine deoxyriboside (0·740 μmol.), 500 μg. of 4-aminoglyoxaline-5-carbonamide. HCl, 500 μl. of 0·2 M-phosphate buffer (pH 8·04), and 500 μl. (3·2 mg.) of *Lb. delbrueckii* enzyme (dialysed for 3 days) were incubated at 37° for 10 hr. (toluene used). Ethanol was added, and a sample of the ethanolic extract was chromatographed in the *n*-butanol-ethyl acetate-water-morpholine system. The control sample showed two spots, one at $R_F = 0·94$ (thymine deoxyriboside) and the other at $R_F = 0·44$ (4-aminoglyoxaline-5-carbonamide). The experimental sample showed three spots, one at $R_F = 0·83$ (presumably thymine), another at $R_F = 0·44$ (4-aminoglyoxaline-5-carbonamide), and a faint spot at $R_F = 0·59$. Microbiological assay of the sheet showed that 0·71 μmol. of thymine deoxyriboside had disappeared. The areas at $R_F = 0·59$ assayed as follows: control, 0·007 μmol., experimental, 0·150 μmol. of deoxyriboside present. The activity thus found by direct assay (0·143 μmol.) accounted for only 20% of the thymine deoxyriboside which disappeared, a result similar to that obtained with the *Lb. helveticus* enzyme (Table 9). There was also considerable drift in the assay of the substance.

Several chromatograms were run, and the control and experimental areas corresponding to $R_F = 0·59$ were collected, cut into small pieces and extracted with water. The solutions were reduced to dryness *in vacuo*. Microbiological assay showed that during the course of this operation the activity had been destroyed, due presumably to hydrolysis in the unbuffered solution. The material thus obtained was re-chromatographed in the *n*-butanol-water-ammonia system.

In the control sample nothing was visible, while the experimental sample showed a dark spot at $R_F = 0·30$. (Found for 4-aminoglyoxaline-5-carbonamide, $R_F = 0·32$.) The two areas were cut out, extracted with water, and the amounts of 4-aminoglyoxaline-5-carbonamide present in the filtrates determined by the Bratton & Marshall (1939) test at $\lambda = 530$; found in the control, 0·002 μmol., and in the experimental, 0·105 μmol.

The filtrate from the control area was placed in the reference cuvette, and the ultraviolet absorption spectrum of the substance was determined. Found, $E_{max.} = 264$ mμ. (Found by Stetten & Fox (1945) for 4-aminoglyoxaline-5-carbonamide, $E_{max.} = 264$ mμ.) Upon the basis of the extinction at 264 mμ. the amount of 4-aminoglyoxaline-5-carbonamide was 0·111 μmol., a value in fair agreement with that found by the Bratton & Marshall test (0·103 μmol.).

These findings are consistent with the idea that 4-aminoglyoxaline-5-carbonamide deoxyriboside was formed by the exchange reaction.

The inactivity of deoxyribose-1-phosphate in the exchange system

Deoxyribose-1-phosphate was tested in this system with the various purines and pyrimidines which entered into the exchange reaction. In no case was any appreciable deoxyriboside formed.

Barium deoxyribose-1-phosphate preparation (8·5 mg.) was dissolved in 1·3 ml. of water. Na_2SO_4 (7 mg.) was added, and the $BaSO_4$ was removed by centrifugation. A sample (600 μl.) of the supernatant and 500 μl. of M-citrate buffer (pH = 3·7) were left at 26° for 10 hr.; 150 μl. of 5 N-NaOH were then added bringing the pH to 7. This hydrolysed solution was used in the control mixture. Another sample of 600 μl. of the supernatant was kept in the frozen state while the first portion was hydrolysing. It was then melted and added last to 650 μl. of the neutralized citrate solution. Samples (125 μl. = 0·474 μmol.) of the deoxyribose-1-phosphate solution or the hydrolysed solution were incubated with 200 μg. of purine or pyrimidine in 200 μl. of 0·1 M-sodium citrate (pH = 7·0), and 200 μl. of *Lb. helveticus* enzyme (dialysed 3 days). The tubes were then heated at 100° for 5 min. The solutions were diluted, filtered, if necessary, and assayed microbiologically. The difference between control and experimental samples was used to calculate the amount of deoxyriboside formed. The authenticity of the deoxyribose-1-phosphate preparation was checked with the calf liver nucleoside phosphorylase system, in which 0·474 μmol. formed 0·36 μmol. of deoxyriboside on incubation with hypoxanthine.

The results (Table 10) show that the *Lb. helveticus* enzyme did not catalyse the synthesis of deoxyribosides from deoxyribose-1-phosphate and the various purines and pyrimidines which enter into the exchange reaction. Moreover, the deoxyribose-1-phosphate preparation did not contain any inhibitor of the *Lb. helveticus* enzyme, since the same amount of exchange took place in the presence of the deoxyribose-1-phosphate as in its absence, and large amounts of phosphate did not inhibit the exchange reaction, although an inhibition might reasonably be expected if deoxyribose-1-phosphate were the intermediate in the exchange reaction.

Table 10. *Inactivity of deoxyribose-1-phosphate in the exchange system with* Lb. helveticus *extract*

(Description in text.)

Purine or pyrimidine added	Deoxyribose-1-phosphate	Hypoxanthine deoxyriboside	Deoxyriboside formed (μmol.)
Hypoxanthine*	+	–	<0·005
Thymine	+	–	<0·005
	+	+	0·11†
	–	+	0·12†
	–	+	0·12†‡

* Also, adenine, guanine, xanthine, 4-amino-5-imidazole carboxamide, and uracil.
† Determined in the usual way following mild acid hydrolysis.
‡ 0·2 M-Phosphate buffer (pH 7·0) used instead of citrate buffer.

There is nothing here to suggest that deoxyribose-1-phosphate plays any role in the exchange reaction.

The inactivity of deoxyribose in the exchange system

If deoxyribose-1-phosphate is dismissed as the intermediate in the interconversion of deoxyribosides, the only reasonable question remaining is whether the reaction involves (1) hydrolysis followed by resynthesis, or (2) trans-*N*-glycosidation. An investigation was made of the comparative amounts of deoxyriboside hydrolysed and of deoxyribose transferred by these enzyme preparations in the same period of time.

Table 11. *The comparative amounts of deoxyriboside hydrolysed and deoxyribose transferred*

		Hydrolysis experiment		Transfer experiment		
		Deoxyriboside hydrolysed			Deoxyribose transferred	
Deoxyriboside tested	μmol. added	(μmol.)	(%)	Purine or pyrimidine added	(μmol.)	(%)
Cytosine deoxyriboside	0·110	—	—	Adenine	−0·004*	−4
Cytosine deoxyriboside	0·110	0·019	17	Adenine	0·097	88
Uracil deoxyriboside	0·088	0·019	23	Adenine	0·083	94
Thymine deoxyriboside	0·103	0·023	22	Adenine	0·085	82
Hypoxanthine deoxyriboside	0·099	0·020	20	Thymine	0·076	76
Guanine deoxyriboside	0·094	0·017	18	Thymine	0·068	72

* Enzyme boiled prior to incubation.

Transfer reaction. 125 μg. of purine or pyrimidine, 125 μl. of 0·1 M-glycine buffer (pH 8·5), 10–15 μl. of deoxyriboside solution and 125 μl. (0·42 mg.) of *Lb. helveticus* enzyme, (dialysed 1 day) were incubated at 37° for 10 hr. The incubation was stopped with 8 μl. of 5 N-HCl. In the control tubes the deoxyribosides were added after the acid had been added. The solutions were hydrolysed, diluted and centrifuged, the supernatant solutions, after neutralization, being assayed as usual.

Hydrolysis. As above, with the purine or pyrimidine omitted. In the case of the purine deoxyribosides, however, the reaction was stopped by heating the incubation mixture at 100° for 5 min., the control tube having the purine deoxyriboside added after the period of heating.

The results (Table 11) show that during a period in which about 80 % of deoxyribose was transferred, about 20 % of the deoxyriboside was split. It might be argued that in the presence of an excess of acceptor, which would combine with one of the products of hydrolysis, an exchange in excess of the hydrolysis in the absence of an acceptor would take place. Thus, 80 % of deoxyribose transferred might not be considered as unreasonable if resynthesis occurred.

The question of resynthesis was next examined. Since crystalline 2-deoxy-D-ribose was not available for testing, a solution of deoxyribose was prepared by the very mild hydrolysis of deoxyribose-1-phosphate. The mixtures were as follows: (1) Thymine (100 μg.) in 100 μl. of 0·1 M-sodium citrate at pH 7·0, 100 μl. of *Lb. helveticus* enzyme (dialysed for 7 hr.; 1·9 mg.). (2) Same as (1) plus 0·24 μmol. of deoxyribose-1-phosphate, hydrolysed at pH 4·0 for 11 hr. at 28° (60 μl. of solution). (3) Same as (2) plus 0·099 μmol. of hypoxanthine deoxyriboside (10 μl. of solution). The tubes were incubated and adjusted to pH 1. To (1) and (2) 10 μl. of hypoxanthine deoxyriboside solution was added, and all tubes were heated at 100° for 5 min., filtered, the filtrates neutralized and assayed microbiologically for pyrimidine deoxyriboside. Found (μmol.): (1) 0·005; (2) 0·005; (3) 0·063. It was hence concluded that the mechanism of the transfer does not involve mere hydrolysis followed by resynthesis.

The presence of the enzyme in other bacteria

Enzyme preparations from two other bacteria, *Lb. delbrueckii* and *Thermobact. acidophilus* R 26, both of which require a deoxyriboside for growth, also catalyse the transfer (Table 12). The enzyme

Table 12. *Exchange reaction using enzymes from* Lb. delbrueckii *and* Therm. acidophilus R 26

(Hypoxanthine deoxyriboside (0·099 μmol.) added as donor.)

Bacterial enzyme	Pyrimidine added	Pyrimidine deoxyriboside formed (μmol.)
Thermobact. acidophilus R 26 (1·1 mg.)	Thymine	0·069
	Uracil	0·057
Lb. delbrueckii (0·81 mg.)	Thymine	0·064
	Uracil	0·061

preparation from *Thermobact. acidophilus* R 26 was tested for its ability to catalyse the synthesis of hypoxanthine deoxyriboside from deoxyribose-1-phosphate plus hypoxanthine. The result was negative: Found, 0·004 μmol. of deoxyriboside formed from 0·474 μmol. of deoxyribose phosphate. The possibility that enzymes of the type described may in some way be related to the requirement of bacteria for deoxyribosides should be further investigated.

An attempt to demonstrate the trans-reaction in a growth experiment with cells of Leuc. citrovorum

This organism responds specifically to thymine deoxyriboside. If, thymine and any of the deoxyribosides to which the organism does not respond in growth are added to the basal medium; if the substances enter the cell, and if a trans-reaction of the above type occurs, thymine deoxyriboside would be formed, and growth would result. A positive result would be consistent with the other experiments. A negative result, of itself, would tell very little: Three deoxyribosides (uracil deoxyriboside, cytosine deoxyriboside and hypoxanthine deoxyriboside) were each tested in combination with thymine. In no case was any growth detected after 28 hr. incubation.

DISCUSSION

Through the use of bacterial enzymes, which catalyse the transfer of the deoxyribosyl group from one purine or pyrimidine to another, it was possible to synthesize certain deoxyribosides, identified by chromatographic methods and microbiological assay, which have not yet been detected as constituents of cells. The questions arise as to whether these deoxyribosides may be components of the bacterial nucleic acid, whether they serve as intermediates in the synthesis of bacterial nucleic acid, or whether they are mere biochemical curiosities.

A considerable number of purines and pyrimidines act as acceptors in the transfer of the deoxyribosyl group, but uric acid, 2:6-diaminopurine, dihydrothymine, and other compounds are inactive. Of the new deoxyribosides which have been detected in this way uracil deoxyriboside is of interest, since it may be envisaged as a precursor of both cytosine deoxyriboside and thymine deoxyriboside. The existence of an enzymic mechanism for the synthesis of 5-methylcytosine deoxyriboside seems reasonable in view of recent work in which it has been established that 5-methylcytosine is a component of the deoxyribonucleic acid of several animal and plant species (Wyatt, 1950). Ever since the original claims made for 4-aminoglyoxaline-5-carbonamide (Shive *et al.* 1947) this compound has been mooted as an intermediate in purine metabolism (Fries, Bergström & Rottenberg, 1949; Greenberg, 1950; Miller, Gurin & Wilson, 1950; Schulman, Buchanan & Miller, 1950; Gotz, 1950). The possibility that 4-aminoglyoxaline-5-carbonamide deoxyriboside may be formed by these bacterial enzymes does not clarify the part it might play in the formation of the bacterial deoxyribonucleic acid. The organism may simply replace the aglycone with appropriate and completely formed purines or pyrimidines which are present in the medium or which the bacterium may synthesize. Such an exchange between thymine and the aglycones of several deoxyribosides was investigated in living cells of *Leuc. citrovorum*. In no case was thymine deoxyriboside formed, but the negative finding is of little value without knowledge of the permeability of the cell to thymine and the deoxyribosides.

Whether enzymes of this type function in the biosynthesis of nucleic acids in higher animals is difficult to assess. The findings with these bacterial enzymes are in contrast with the data showing that pyrimidines are not incorporated into the nucleic acids of rats, while the cytosine of cytidine (Hammarsten, Reichard & Saluste, 1950) and the cytosine and thymine of the corresponding deoxyribosides are incorporated. It would be of interest to know, whether the ribose or deoxyribose of the administered nucleoside is distributed among all of the purines and pyrimidines of the nucleic acids, for which purpose nucleosides labelled not in the pyrimidine- but in the sugar-portion of the molecules would be required, and whether the bacterial enzymes will catalyse the transfer of purines and pyrimidines in polynucleotides.

The remarkable rapidity with which the enzyme preparations split the ribosides is a technical difficulty which must be first solved before any trans-type reaction can be demonstrated in this series by use of these enzymes—if, indeed, such occurs (Kalckar *et al.* 1951).

Since both deoxyribose-1-phosphate and deoxyribose (the only two compounds which might reasonably be expected to act as intermediates) are inactive in the exchange system the enzyme is considered to be a trans-*N*-glycosidase. The similarity of these reactions to those catalysed by the transglucosidase from *Pseudomonas saccharophila* (Doudoroff *et al.* 1947) is most remarkable, although alcoholysis (and phosphorolysis) of glucose-1-phosphate is catalysed by that enzyme preparation, whereas, in the system here described, aminolysis of deoxyribose-1-phosphate does not occur. A β-glucosidase from *Salix purpurea* L. has been described which catalyses the transfer of the glucosyl group from phenols to primary alcohols (Rabaté, 1935). 'Trans-pentosidation' from guanine to hypoxanthine has been claimed (Kritskii, 1950). A trans-phosphorylase catalyses the transfer of the phosphoryl group from phenols to primary alcohols (Axelrod, 1948), and the same type of reaction has been extended to include the phosphorylation of glycerol by creatine phosphate (Meyerhof & Green, 1950). A trans-peptidase has been reported (Hanes, Hird & Isherwood, 1950) which catalyses the aminolysis of γ-glutamyl peptides by amino-acids. In analogy with this trans-peptidase one may suppose that esterases which catalyse the transfer of acyl

groups from one alcohol to another will be discovered when a serious search is made for them.

Since lactose and other β-galactosides but not galactose serve equally well as the substrate for *Lactobacillus bulgaricus* in a medium containing glucose (Snell, Kitay & Hoff-Jørgensen, 1948) the interesting problem is presented as to whether a trans-β-galactosidase may function in the nutrition of certain bacteria. No completely satisfying explanation of how such trans-reactions are brought about has yet been devised. It is becoming apparent that trans-reactions play a more general role in biological processes than has been previously supposed.

SUMMARY

1. Dialysed enzyme preparations from *Lactobacillus helveticus* have been shown, by indirect methods, to catalyse the transfer of the deoxyribosyl group from one purine or pyrimidine to another.

2. By these methods, uracil deoxyriboside, thymine deoxyriboside, 5-methylcytosine deoxyriboside, and cytosine deoxyriboside were shown to be formed by the interaction of the corresponding pyrimidine and purine deoxyribosides. Orotic acid was not tested.

3. Conversely, substances which showed the lability to mild acid hydrolysis characteristic of purine deoxyribosides were formed when pyrimidine deoxyribosides reacted enzymically with adenine, guanine, hypoxanthine, xanthine, and 4-aminoglyoxaline-5-carbonamide. Uric acid, 2:6-diaminopurine, and certain other compounds were inactive.

4. The transfer was catalysed in phosphate-free buffers by enzyme preparations which had been dialysed for several days.

5. Neither deoxyribose nor deoxyribose-1-phosphate was active in the transfer reaction.

6. The enzyme involved is considered to be a trans-*N*-glycosidase, analogous to the trans-*O*-glycosidase from *Pseudomonas saccharophila* (Doudoroff *et al.* 1947).

7. Enzyme preparations from two other deoxyriboside-requiring bacteria (*Lactobacillus delbrueckii* and *Thermobacterium acidophilus* R 26) also catalysed the transfer.

8. An attempt to demonstrate the trans-reaction in living cells of *Leuconostoc citrovorum* was unsuccessful.

Grateful acknowledgement is made of the many helpful suggestions, interesting discussion, and kindnesses which were extended to me during the course of this work by Dr H. M. Kalckar and Dr E. Hoff-Jørgensen, in whose laboratories the present investigation was carried out. I should like to express my appreciation to Mrs A. L. Ziesler for technical assistance, and also to Mr H. Klenow and Dr A. Kjaer for their helpfulness, Dr O. G. Bentley and Dr T. Jukes for bacterial cultures, Mr H. Kihara for various supplies, Miss Fredsted, Miss Ruth Schwartz and Mrs Audrey Jarnum for assistance with some of the microbiological work.

This work was supported by grants from Lederle Laboratories Division, American Cyanamid Co., Carlsbergfondet, and The Rockefeller Foundation.

REFERENCES

Axelrod, B. (1948). *J. biol. Chem.* **172**, 1.
Bereblum, I. & Chain, B. (1938). *Biochem. J.* **32**, 295.
Bratton, A. C. & Marshall, E. K. jun. (1939). *J. biol. Chem.* **128**, 537.
Davis, A. R., Newton, E. B. & Benedict, S. R. (1922). *J. biol. Chem.* **54**, 598.
Doudoroff, M., Barker, H. A. & Hassid, W. Z. (1947). *J. biol. Chem.* **168**, 725.
Friedkin, M. (1950). *J. biol. Chem.* **184**, 451.
Friedkin, M. & Kalckar, H. M. (1950). *J. biol. Chem.* **184**, 437.
Fries, N., Bergström, S. & Rottenberg, M. (1949). *Physiologia Plantarum*, **2**, 210.
Gotz, J. S. (1950). *Arch. Biochem.* **29**, 222.
Greenberg, G. R. (1950). *Fed. Proc.* **9**, 179.
Hammersten, E. (1924). *Biochem. Z.* **144**, 383.
Hammarsten, E., Reichard, P. & Saluste, E. (1950). *J. biol. Chem.* **183**, 105.
Hanes, C. S., Hird, F. J. R. & Isherwood, F. A. (1950). *Nature, Lond.*, **166**, 288.
Hoff-Jørgensen, E. (1949). *J. biol. Chem.* **178**, 525.
Hoff-Jørgensen, E. (1952). *Biochem. J.* **50**, 400.
Kalckar, H. M. (1947). *J. biol. Chem.* **167**, 477.
Kalckar, H. M., Kjeldgaard, N. O. & Klenow, H. (1950). *Biochem. Biophys. Acta*, **5**, 575.
Kalckar, H. M., MacNutt, W. S. & Hoff-Jørgensen, E. (1952). *Biochem. J.* **50**, 397.
Kitay, E., MacNutt, W. S. & Snell, E. E. (1949). *J. biol. Chem.* **177**, 993.
Kitay, E., MacNutt, W. S. & Snell, E. E. (1950). *J. Bact.* **59**, 727.
Klein, W. (1938). *Hoppe-Seyl. Z.* **255**, 82.
Kocher, V. & Schindler, D. (1949). *Int. Z. Vitaminforsch.* **20**, 441.
Kritskii, G. A. (1950). *Dokl. Akad. Nauk. U.S.S.R.* **70**, 667.
Levene, P. A. (1921). *J. biol. Chem.* **48**, 122.
Levene, P. A. & Bass, L. W. (1931). *Nucleic Acids*, p. 145. New York: The Chemical Catalog Company Inc.
Levene, P. A. & London, E. S. (1929). *J. biol. Chem.* **83**, 793.
MacNutt, W. S. (1950). *Nature, Lond.*, **166**, 444.
MacNutt, W. S. & Snell, E. E. (1950). *J. biol. Chem.* **182**, 557.
Markham, R. & Smith, J. D. (1949). *Biochem. J.* **45**, 294.
Meyerhof, O. & Green, H. (1950). *J. biol. Chem.* **183**, 377.
Miller, C. S., Gurin, S. & Wilson, D. W. (1950). *Science*, **112**, 654.
Ploeser, J. McT. & Loring, H. S. (1949). *J. biol. Chem.* **178**, 434.

Rabaté, M. J. (1935). *Bull. Soc. Chim. biol., Paris*, **17**, 572. 654.
Schmidt, G. & Thannhauser, S. J. (1943). *J. biol. Chem.* **149**, 369.
Schulman, M. P., Buchanan, J. M. & Miller, C. S. (1950). *Fed. Proc.* **9**, 225.
Shaw, E. & Woolley, D. W. (1949). *J. biol. Chem.* **181**, 89.
Shive, W., Ackermann, W. W., Gordon, M., Getzendaner, M. E. & Eakin, R. E. (1947). *J. Amer. chem. Soc.* **69**, 725.
Snell, E. E., Kitay, E. & Hoff-Jørgensen, E. (1948). *Arch. Biochem.* **18**, 494.
Stetten, M. R. & Fox, C. L. (1945). *J. biol. Chem.* **161**, 333.
Wyatt, G. R. (1950). *Nature, Lond.*, **166**, 237.

Trans-*N*-Glycosidase Studied with Radioactive Adenine

By H. M. KALCKAR, W. S. MACNUTT* and E. HOFF-JØRGENSEN
Institutes of Cell Physiology and Biochemistry, University of Copenhagen

(*Received* 19 *February* 1951)

It has been shown (MacNutt, 1952) that extracts from *Lactobacillus helveticus* contain an enzyme—in effect a trans-*N*-glycosidase—which catalyses an exchange between purines incorporated in the deoxyribosidic linkage and free pyrimidines, and the converse exchange between a pyrimidine deoxyriboside and a free purine. The same type of exchange was also shown to take place between one purine of a purine deoxyriboside and another purine base; e.g. adenine + guanine deoxyriboside ⇌ guanine + adenine deoxyriboside.

In the closely related reaction demonstrated by MacNutt (1952): adenine + hypoxanthine deoxyriboside (HDR) ⇌ hypoxanthine + adenine deoxyriboside (ADR), the formation of adenine nucleoside could be the result of a transamination, involving only the 6-amino group of the free adenine. Alternatively, the reaction could be due to a transglycosidase involving transfer of the whole adenine molecule. The mechanism of the reaction has now been investigated using adenine labelled at carbon atom number 8 with ^{14}C, and determining the isotope concentration in the reaction products. The two types of reactions can be illustrated as follows:

(1) *Transamination*

[^{14}C]-adenine + HDR ⇌ [^{14}C]-hypoxanthine + ADR.

In this case the free hypoxanthine contains undiluted isotopic carbon, and the adenine nucleoside does not contain any excess isotopic carbon.

(2) *Trans-N-glycosidase*

[^{14}C]-adenine + HDR ⇌ hypoxanthine + [^{14}C]-ADR.

In this case the radioactivity of the reaction products will be found exclusively in the adenine nucleoside, in which the isotopic concentration will be as high as that of the radioactive adenine added, while the free hypoxanthine will be devoid of radioactivity.

* Present address: University Chemical Laboratory, Cambridge, England.

EXPERIMENTAL

Materials

Adenine labelled in position 8 with ^{14}C, was made from [^{14}C]-formic acid and 4:5:6-triaminopyrimidine (Clark & Kalckar, 1950). The preparation used contained about 0·8 mc./μmol.

Commercial preparations of *hypoxanthine* were used.

Hypoxanthine deoxyriboside was prepared from enzymically degraded thymus nucleic acid as described by MacNutt (1952).

Adenosine deoxyriboside was supplied as the monohydrate through the kindness of Dr T. G. Brady, University of Dublin, Ireland (Brady, 1941).

Adenosine was obtained either from Fordomes Trading Company, Arlington, N.J., U.S.A., or from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.

Inosine was prepared from adenosine by enzymic deamination (Kalckar, 1947*a*).

The enzyme preparation from *Lb. helveticus* was that described by MacNutt (1952).

Methods

Chromatography with *n*-butanol-water-ammonia or *n*-butanol-water-ethyl acetate-morpholine was performed as described by MacNutt (1952). Hypoxanthine was determined by addition of xanthine oxidase and recording the increase in density at 293 mμ. (Kalckar, 1947*a*).

Adenine was characterized by its R_F value in paper chromatography and determined by microbiological assay with *Lb. casei* (Roberts & Snell, 1946). Since this assay, although quite accurate from a quantitative point of view, is neither specific nor sensitive, the optical method for the determination of adenine described by Klenow (1952) was used.

Deoxynucleosides were measured by a microbiological assay using *Thermobacterium acidophilus* (Hoff-Jørgensen, 1952) and differentiated by chromatography on paper (MacNutt, 1952) and by enzymic ultraviolet spectrophotometry (Kalckar, 1947*a*, *b*). The procedure for adenosine deoxyriboside (ADR) was as follows:

The total amount of deoxyriboside present (e.g. in an eluate from a chromatogram spot) was determined by microbiological assay. The ADR content was determined spectro-

photometrically by addition of intestinal deaminase and measurement of the decrease in density at 265 mμ. (Kalckar, 1947*b*); this estimation was checked by determination of the hypoxanthine deoxyriboside (HDR) formed, by means of the spectrophotometric assay using liver nucleoside phosphorylase and xanthine oxidase (Kalckar, 1947*a*; Friedkin & Kalckar, 1950). The second method frequently gave lower values than the first. Both spectrophotometric determinations, especially that of HDR, were greatly hampered by the presence of some strongly absorbing materials in the eluate, presumably a breakdown product of morpholine. Upon addition of adenosine deaminase or xanthine oxidase to eluates of filter paper treated with this solvent alone, the density at 265 mμ. did not change as a result of enzymic conversion of the unknown absorbing material. It would, nevertheless, be advisable in future to attempt to remove these disturbing substances.

RESULTS

Enzymic interaction of [^{14}C]*-adenine and hypoxanthine deoxyriboside*

The experimental mixture contained 500 μg. of [^{14}C]-adenine. HCl.$\frac{1}{2}H_2O$ in 500 μl. of 0·2M-phosphate buffer at pH 7·0, 50 μl. of HDR (125 μg., 0·496 μmol.) and 500 μl. of *Lb. helveticus* extract (dialysed 3 days; 1·7 mg. dry weight. The control mixture was the same except that the HDR was added after the period of incubation had been terminated by the addition of ethanol. The mixtures were incubated for 12 hr. at 37° (1 drop of toluene added) and deproteinized by addition of 7 ml. of ethanol. The ethanolic extracts were prepared as described previously (MacNutt, 1952). Final volume = 500 μl.

First chromatographic separation. The entire volumes were chromatographed in the *n*-butanol-water-ammonia system. For each paper chromatogram 100 μl. of ethanolic extract were placed on paper along 4 cm., using adenine and HDR as indicators. Adenine and ADR appeared at one spot ($R_F = 0·40$), hypoxanthine and HDR at the other spot ($R_F = 0·18$). The appropriate spots from several chromatograms were pooled, cut into small pieces and extracted with water.

The filtered solutions from the ADR-adenine spots were assayed microbiologically. The experimental sample contained ADR equivalent to 0·365 μmol. in the total incubation mixture, while the control sample contained none. This yield corresponds to a conversion of 74% of the initial HDR. HDR and hypoxanthine were not determined until after these filtrates had been rechromatographed.

Second chromatographic separation. ADR was then separated from adenine, and HDR from hypoxanthine, by chromatography of the separate filtrates in the *n*-butanol-water-morpholine-ethyl acetate system (MacNutt, 1952). The individual spots were cut out and extracted with water as before. The volume of filtrate in each case was 4·0 ml. Hypoxanthine was estimated by the increase in density at 293 mμ. upon addition of xanthine oxidase and HDR and ADR by microbiological assay. The results were as follows:

	μmol./4 ml. eluate		
Sample	ADR	HDR	Hypoxanthine
Control	0·005	0·132	0·063
Experimental	0·241	0·004	0·140

Radioactivity of the filtrates from the second chromatogram. Samples of 50, 100 and 150 μl. were dispensed from constriction pipettes on to clean aluminium dishes and dried beneath an infrared lamp to form areas of about 4 mm. in diameter. Radioactivity counts were taken. The results were averaged and calculations made upon the basis of the counts above background per μg. of purine. ADR and adenine were expressed as $C_5H_5N_5$. HCl.$\frac{1}{2}H_2O$. The results set out in Table 1 show unambiguously (1) that free adenine can be exchanged with the hypoxanthine in HDR, and (2) that in this enzyme system no transamination between the 6-amino group of adenine and HDR took place; otherwise the adenine in the ADR which was formed should have shown a lower ^{14}C concentration than that of the free [^{14}C]-adenine.

Table 1. *Enzymic formation of* [^{14}C]*-adenine deoxyriboside from hypoxanthine deoxyriboside and* [^{14}C]*-adenine*

Sample analysed		Purine for counting (μg.)	Counts/min.	Counts/min./μmol. purine (× 10^4)
Eluate from ADR spot	Control	0·010	5·0	—
	Experimental	0·273	100·8	6·62
		0·546	208·0	6·88
Eluate from HDR spot	Control	0·200	1	—
	Experimental	0·006	1	—
Eluate from hypoxanthine spot	Control	0·100	1·0	—
		0·200	1·2	—
Standard [^{14}C]-adenine	—	0·250	91·2	6·60
		0·500	183·6	6·63

Enzymic exchange of free [^{14}C]*-adenine with the adenine of adenine deoxyriboside*

It was considered of interest to investigate whether the adenine of ADR would be exchanged with free [^{14}C]-adenine in the presence of the bacterial enzyme, for a reaction of this type would raise problems related to those encountered in the enzymic exchange of ^{32}P with the phosphorus of glucose-1-phosphate in the absence of added sugars (Doudoroff, Barker & Hassid, 1947). It was found indeed that on incubation of ADR with the bacterial enzyme and a fivefold excess of [^{14}C]-adenine the exchange took place and was practically complete after 10·5 hr. incubation. The enzyme used, however, contained traces of hypoxanthine, which might have acted as a 'carrier' of the deoxyribosyl group through the reactions previously established:

ADR + hypoxanthine $\leftrightarrows$ HDR + adenine,

HDR + [^{14}C]-adenine $\leftrightarrows$ [^{14}C]-ADR + hypoxanthine.

It is, therefore, not yet clear if a direct exchange takes place; further work with an ezyme preparation free from potential 'carriers' is necessary to clarify this point.

Incubation of [^{14}C]*-adenine with adenine riboside and* Lb. helveticus

Stephenson & Trim (1938) showed that *Escherichia coli* cells catalysed the deamination of adenine to hypoxanthine provided that either adenosine (or inosine) was present in appreciable amounts. Since the amount of the purine nucleoside required was only 10–20 % of the amount of adenine metabolized and the adenosine was rapidly deaminated, they concluded that the nucleoside acts as a transfer system. They formulated the reaction as a transamination:

(1) adenine + inosine = hypoxanthine + adenosine,

(2) adenosine = inosine + ammonia.

We were interested to study this reaction of riboside compounds which could be either a transamination or a trans-*N*-glycosidase type of reaction as it is analogous to the reaction with deoxyribosides.

Attempts were made to study the reaction using *Lb. helveticus* as a source of the enzyme, since *Esch. coli* extracts are rich in purine riboside phosphorylase (Kalckar, unpublished) and were, therefore, considered unsuitable for this purpose.

Mixtures were made as follows: *Experimental sample*: 100 μl. (212 μg.) [^{14}C]-adenine.HCl.$\frac{1}{2}H_2O$, 250 μl. 0·067 M-phosphate buffer at pH 8·3, 250 μl. of dialysed *Lb. helveticus* enzyme (0·8 mg. dry weight) and 50 μl. (57 μg.) adenosine. *Control* (1): same as the experimental sample except that the adenosine was added after the incubation period had been terminated by the addition of ethanol. *Control* (2): same as the experimental sample except that the enzyme was added after incubation. The samples were incubated at 35° for 2 hr. at which time 7 ml. of ethanol were added, and the ethanolic extracts prepared in the usual way. The extracts were chromatographed in the *n*-butanol-water-ammonia system.

The chromatogram showed the following phenomena: (*a*) there was neither an adenosine nor an inosine spot in the experimental sample; (*b*) in the experimental sample there was a strong hypoxanthine spot which was devoid of radioactivity; (*c*) all the adenine spots were strongly radioactive; (*d*) the two controls showed highly radioactive adenine spots (R_F, 0·37) and non-radioactive adenosine spots (R_F, 0·30).

It was concluded, therefore, that adenosine had been deaminated and that the inosine (and perhaps the adenosine) had been completely split within 2 hr. by a ribosidase. No exchange occurred during this time, since the hypoxanthine was devoid of radioactivity.

It appears that extracts of *Lb. helveticus* contain a ribosidase and also a deaminase which catalyses the deamination of adenosine but not (according to the earlier experiments) adenine deoxyriboside. This is in contrast to the action of the mammalian adenosine deaminase as well as the deaminases of *Esch. coli* and *Lb. casei*, which bring about a deamination of both adenine deoxyriboside and adenosine.

SUMMARY

1. The exchange between adenine and the hypoxanthine of hypoxanthine deoxyriboside catalysed by an enzyme preparation from *Lactobacillus helveticus* has been studied. Using [^{14}C]-adenine, the reaction gave rise to adenine deoxyriboside with a molar ^{14}C concentration as high as that of the initial adenine.

2. The reaction is therefore not a transamination involving the 6-positions of adenine and hypoxanthine deoxyriboside, but is probably an exchange of the adenine skeleton with the bound hypoxanthine, catalysed by a trans-glycosidase.

3. The enzyme preparation catalysed an exchange reaction between the adenine of adenine deoxyriboside and [^{14}C]-adenine. It also rapidly degraded adenine riboside (adenosine) by hydrolysis and deamination, but did not deaminate adenine deoxyriboside.

Our thanks are due to the following foundations: Carlsbergfondet, The Rockefeller Foundation, Lederle Laboratories Division, American Cyanamid, for grants which made this work possible. One of us (W.S.M.) is indebted to the Jane Coffin Childs Memorial Fund for a fellowship.

The authors take pleasure in expressing their thanks to Dr T. G. Brady, University of Dublin, for his generous gift of adenine deoxyriboside, to Miss I. Fredsted for valuable help in the microbiological determinations and to Prof. E. Lundsgaard and Dr P. Kruhöffer for their hospitality and willingness to put laboratory space and equipment at our disposal.

REFERENCES

Brady, T. G. (1941). *Biochem. J.* **35**, 855.
Clark, V. M. & Kalckar, H. M. (1950). *J. chem. Soc.* p. 1029.
Doudoroff, M., Barker, H. A. & Hassid, W. Z. (1947). *J. biol. Chem.* **168**, 725.
Friedkin, M. & Kalckar, H. M. (1950). *J. biol. Chem.* **184**, 437.
Hoff-Jørgensen, E. (1952). *Biochem. J.* **50**, 400.
Kalckar, H. M. (1947*a*). *J. biol. Chem.* **167**, 429.
Kalckar, H. M. (1947*b*). *J. biol. Chem.* **167**, 445.
Klenow, H. (1952). *Biochem. J.* **50**, 404.
MacNutt, W. S. (1952). *Biochem. J.* **50**, 384.
Roberts, E. C. & Snell, E. E. (1946). *J. biol. Chem.* **163**, 499.
Stephenson, M. & Trim, A. R. (1938). *Biochem. J.* **32**, 470.

A Microbiological Assay of Deoxyribonucleosides and Deoxyribonucleic Acid

By E. HOFF-JØRGENSEN
Institute of Biochemistry, University of Copenhagen, Denmark

(*Received* 19 *February* 1951)

In the course of a survey of the nutritional requirements of certain lactic acid bacteria a strain, *Thermobacterium acidophilus* R 26, was encountered whose requirement for a deoxyriboside could not be replaced either by vitamin B_{12} or by commercial antipernicious anaemia preparations from liver. This organism, therefore, seemed well suited as a test organism for a microbiological assay of deoxyribonucleic acid (DNA) more specific and more sensitive than the methods of Schmidt & Thannhauser (1945) and Schneider (1945). The method described below allows the determination of a few μg. of DNA with an accuracy of about 10 %, even in tissues, such as frogs eggs, where the concentration of DNA is less than 0·01 %.

EXPERIMENTAL

Methods

Stock cultures are maintained by weekly transfer in skimmed milk to which is added 0·1 % cysteine and 0·5 % 'Difco' yeast extract. The tubes are autoclaved for 10 min. at 120° and stored at about 10°.

Inoculum medium. Double-strength basal medium (100 ml.) and 100 ml. of 0·02 M-deoxyriboside are mixed and autoclaved in 5 ml. portions for 10 min. at 120°.

Inoculum. A small loopful of the stock milk culture is transferred to a tube containing 5 ml. of the inoculum medium. After incubation at 37° for 20–24 hr., the cells are centrifuged, washed once with 10 ml. of sterile saline, and resuspended in 10 ml. of sterile saline. One small drop of this suspension is used to inoculate each assay tube.

Standard. Stock solution: 10^{-4} g.mol. of deoxyriboside, e.g. 24·2 mg. of thymidine, is dissolved in 100 ml: 25 % ethanol. This solution is stable for at least 1 year. To make a working standard containing 5×10^{-9} g.mol. of deoxyriboside/ml., 0·050 ml. of the stock solution is diluted to 10 ml. with water.

Table 1. *Double-strength basal medium*

Component	Amount (g./100 ml.)
HCl-hydrolysed casein*	2·5
Enzyme-hydrolysed casein†	0·5
Sodium acetate	3·0
Glucose	3·0
'Tween 80'	0·1
DL-Cysteine	0·05
DL-Tryptophan	0·02
KH_2PO_4	0·2
K_2HPO_4	0·2
$MgSO_4.7H_2O$	0·02
$MnSO_4.4H_2O$	0·004
$FeSO_4.7H_2O$	0·002
	(mg./100 ml.)
Adenine	2
Guanine	2
Thymine	2
Cytidylic acid	5
Riboflavin	0·1
Nicotinic acid	0·1
Calcium pantothenate	0·1
p-Aminobenzoic acid	0·1
Pteroylglutamic acid adjusted to pH 6·8 with NaOH	0·005

* Prepared according to Snell (1950), neutralized with NH_3 instead of NaOH.
† Prepared according to Roberts & Snell (1946).

Basal medium. The composition of the basal medium is shown in Table 1. The addition of the following substances to the basal medium had no effect on the growth curve: ribonucleic acid, depolymerized ribonucleic acid, ribose, asparagine, ascorbic acid (20 mg./100 ml.); adenosine, adenylic acid, uracil, uridine, guanine, guanylic acid, cytosine, cytidine, oleic acid, deoxyribose, deoxyribose-1-phosphate, choline, inositol, cadaverine, putrescine (2 mg./100 ml.); aneurin, biotin (0·1 mg./100 ml.); vitamin B_{12} (1 μg./100 ml.); *Citrovorum* factor (approx. 0·1 mg./100 ml.) and cozymase (1 mg./100 ml.).

Assay procedure. The tubes used for the assay are ordinary rimless Pyrex tubes 100 × 12 mm. The internal diameter of the tubes should not differ by more than 1 mm. at most. The standard working solution is added in amounts of 0·0, 0·1, 0·2, 0·4, 0·6, 0·8 and 1·0 ml. in duplicate. The sample is usually added in amounts of 0·2, 0·4, 0·6 and 0·8 ml. The contents of the tubes are diluted to 1 ml. with water and 1 ml. of the double-strength basal medium is added.

The solutions of the samples and the standard should be measured with an accuracy of 2% or better. The tubes are covered with caps and shaken to ensure thorough mixing. After autoclaving for 5 min. at 120° the tubes are cooled to room temperature, inoculated and incubated at 37° for 24 hr.

Determination of response. The tubes are shaken vigorously, and the contents are transferred to a round Coleman cuvette, type 6-310 B (10 × 75 mm.). The turbidity is read in a photoelectric colorimeter. We have used the apparatus described by Rehberg (1943). 100 = reading of the instrument with uninoculated medium, 0 = complete darkness.

The results are calculated from the standard dose-response curve (Fig. 1) according to Snell (1950) and expressed in g.mol. of deoxyriboside. 1 g.mol. deoxyriboside is equivalent to 327 g. DNA.

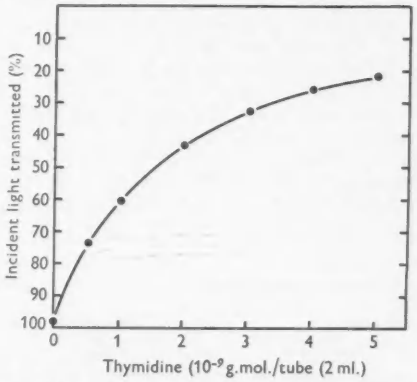


Fig. 1. The growth curve with thymidine (*Thermobacterium acidophilus* R 26). Total volume = 2 ml./tube.

The growth effect of different deoxyribosides

The growth effect of several deoxyribosides was compared, using a sample of thymidine, recrystallized three times, as a standard. The figures in Table 2 indicate that within the experimental error all the deoxyribosides tested have the same growth effect. After boiling at pH 2 for 5 min. the growth effect of the purine deoxyribosides completely disappeared, while the effect of the pyrimidine deoxyribosides was unimpaired.

Table 2. *The growth effect of some deoxyribosides*

Deoxyriboside (equimolar concentrations)	Growth effect	Growth effect after boiling at pH 2 for 5 min.
Thymidine*	100	100
Thymidine†	88, 90, 94	90
Thymidine‡	101, 98, 87	96
Guanine deoxyriboside§	104, 96, 96	0
Guanine deoxyriboside*	95, 97, 100	0
Adenine deoxyriboside‖	106, 108, 112	0
Cytosine deoxyriboside*	98, 106, 105	104
Hypoxanthine deoxyriboside*	92, 96, 90	0

* Prepared by Dr W. S. MacNutt in this Institute.
† Gift from Dr R. Hotchkiss, Rockefeller Institute for Medical Research.
‡ Gift from Dr W. Shive, University of Texas.
§ Prepared by Dr M. Friedkin in the Institute of Cytophysiology, Copenhagen.
‖ Gift from Dr G. O. Brady, University of Dublin.

Comparison of the growth effect of DNA, deoxyribonucleotides and deoxyribonucleosides

A solution (*a*) of DNA was prepared by dissolving 1 mg. of sodium thymonucleate (Hammarsten, 1924) (P = 9·15%, N = 15·97%) in 5 ml. of phosphate buffer pH 6·8.

A solution of deoxyribonucleotides (*b*) was prepared by incubating 1 ml. of the DNA solution (*a*) and 0·1 ml. 0·01 M-$MgSO_4$ with 100 μg. crystalline deoxyribonuclease (obtained from Worthington Biochemical Laboratory Freehold, New Jersey, U.S.A.) for 10 hr. at 37°. A solution of deoxyribonucleosides was prepared by incubating 0·5 ml. of solution (*b*) with 0·1 ml. intestinal phosphatase (Schmidt & Thannhauser, 1943) for 10 hr. at 37°.

From the results shown in Table 3 it appears that DNA prepared according to Hammarsten (1924) has a growth effect for *Thermobact. acidophilus* R 26 which is less than 1% of the effect of the amount of deoxyribonucleosides present in the DNA.

If, however, the DNA is depolymerized by deoxyribonuclease without liberation of inorganic phosphate (Kunitz, 1950), the growth response is maximum, i.e. the effect is equivalent to the effect of the calculated content of deoxyribosides in the DNA (70·6%). The release of inorganic phosphate by

Table 3. *The growth effect for* Thermobact. acidophilus R 26 *of DNA, deoxynucleotides and deoxynucleosides*

Growth factor	Content of deoxynucleoside (10^{-6} g.mol./mg. DNA): Found by growth effect	Calculated*
DNA	0·021, 0·020, 0·022, 0·022	3·06
DNA, depolymerized	2·95, 2·85, 2·85, 3·15	3·06
DNA, depolymerized and dephosphorylated	3·20, 3·00, 3·30, 2·85	3·06

* Assuming 1 g.mol. deoxynucleoside ~ 327 g. DNA.

intestinal phosphatase is not associated with any additional increase or any decrease in the growth effect. Deoxyribonucleotides therefore have the same effect as deoxyribonucleosides in equimolar concentrations.

The determination of DNA

A piece of tissue containing at least 0·3 μg. P as DNA is placed in a small test tube. After addition of an exactly measured amount of 0·5N-sodium hydroxide (at least 5 μl./mg. of fresh tissue) the tube is placed in a boiling-water bath for 15 min. During this time the tissue is disintegrated with a glass rod. After the incubation at 100° 5 vol. of a solution containing 0·06 g.mol. of maleic acid and 0·01 g.mol. of magnesium sulphate per l. are added for each vol. of 0·5N-sodium hydroxide used above. After addition of 5 μg. of crystalline deoxyribonuclease/mg. fresh tissue the tube is incubated at 37° for at least 16 hr. The mixture is then diluted to contain about 3×10^{-9} g.mol. deoxyriboside/ml., centrifuged if necessary, and assayed.

Samples of bacteria, yeast or tissue may either be analysed in the wet state or after drying with acetone. For the analysis of bacteria and yeast, the cells should be disintegrated, preferably in 'the tuning fork disintegrator' (obtained from H. Mickle, Middlesex, England).

Accuracy of the method. An assay of DNA in 0·5 mg. dry *Lactobacillus casei* cells was repeated ten times within 2 months. The mean value found was $1·25 \times 10^{-6}$ g.mol. deoxyriboside/100 mg. dry cell. The standard deviation was $0·08 \times 10^{-6}$ g.mol.

Comparison of the results obtained with two different methods for the content of DNA in some tissues. A male rat, weight 230 g., was killed by decapitation. The tissues were analysed immediately by the microbiological method described in this paper and by the method of Schmidt & Thannhauser (1945). Table 4 shows that the results of the two methods agree reasonably well.

Determination of DNA in tissues, etc., which contain very little DNA. The results of the microbiological assay of the DNA in tissues which contain less than about 0·1 % DNA cannot be verified by comparison with the results obtained by other methods, as no other method allows the determination of DNA in such tissues. The validity of the results given in Table 5 is, however, supported by the facts that added amounts of DNA are recovered, and that about half the growth effect is destroyed by mild acid hydrolysis of samples in which the DNA has been depolymerized.

The content of DNA in certain bacteria. Mixtures of 50 ml. of the basal medium described on p. 400 and 50 ml. of water containing varying amounts of thymidine were inoculated and incubated for 24 hr. at 37°. The cells were spun down, washed with distilled water, treated twice with 10 ml. of acetone and dried in a vacuum over sulphuric acid. The content of DNA in the cells and the residual deoxyriboside in the medium was then determined. It is seen from Table 6 that with both *Thermobact. acidophilus* R 26 and *Thermobact. lactis* I the amount of thymidine taken up from the medium is quantitatively recovered from the cells as DNA.

Table 4. *Estimation of DNA in rat tissues by two methods*

Tissue	Microbiological assay: 10^{-3} g.mol. deoxyriboside/100 g. fresh wt.	Microbiological assay: DNA-P* mg./100 g. fresh wt.	Method of Schmidt & Thannhauser (mg. DNA-P/100 g. fresh wt.)
Liver	1·13	35	31
Kidney	1·20	37	33
Testes	0·95	30	24
Spleen	3·9	121	114
Brain	0·33	10·2	13

* DNA-P calculated from the microbiological assay assuming 1 atom P/mol. deoxyriboside.

Table 5. *Content of DNA in tissue etc. very low in DNA*

Tissue, etc.	Added DNA (mg./100 g.)	Deoxyriboside found as DNA (mg./100 g.)	Deoxyriboside found as DNA after acid hydrolysis* (mg./100 g.)
Rat muscle	0	43	20
	50	91	42
Frog eggs	0	6·5	3·4
	5	11·3	5·4
Tobacco mosaic virus	0	62	24
	60	130	60

* pH 1, 100°, 5 min. after depolymerization.

Table 6. *The content of DNA in two lactic acid bacteria*

(100 ml. of medium.* 24 hr. at 37°.)

Organism	Thymidine added (10^{-6} g.mol.)	Yield of dry cells (mg.)	Deoxyriboside (10^{-6} g.mol.) As DNA in the cells	Residual in medium	Total recovered
Thermobact. acidophilus R 26	0·2	25·9	0·16	0·03	0·19
Thermobact. acidophilus R 26	0·4	31·9	0·35	0·03	0·38
Thermobact. acidophilus R 26	0·8	38·5	0·72	0·04	0·76
Thermobact. lactis I†	0·5	49·6	0·45	0·03	0·48
Thermobact. lactis I	1·0	95·2	0·86	0·07	0·93

* Basal medium (50 ml.) as described in Table 1 and 50 ml. of water.
† This organism will grow either on vitamin B_{12} or on a deoxyriboside.

DISCUSSION

The method presented in this paper is based on the finding that deoxyribonucleosides are essential growth factors for *Thermobact. acidophilus* R 26.

The sensitivity of the method is limited only by the requirement of the organism for deoxyribonucleosides. In a medium containing all other essential and stimulatory growth factors half maximum growth is produced by about 10^{-9} mole deoxyriboside/ml., corresponding to about 0·3 μg. DNA/ml. As no other substances seem to interfere with the assay, analysis can be made of tissues etc. which are either very poor in DNA or of which only small amounts can be obtained.

The essential requirement of *Thermobact. acidophilus* R 26 for a deoxyribonucleoside is elucidated by the finding that the amount of DNA found in the cells is equal to the amount of deoxyribonucleoside taken up from the medium. As the organism will grow equally well on each one of at least five different deoxyribonucleosides, some process in the cell must take place by which the bound deoxyribose taken up from the medium is distributed among the four different purine- and pyrimidine-deoxyribosides found in the DNA. Such a mechanism has been demonstrated in some deoxyriboside-requiring organism by Kalckar, MacNutt & Hoff-Jørgensen (1952).

SUMMARY

1. A microbiological method for the determination of deoxyribosides and deoxyribonucleic acid has been devised, using *Thermobacterium acidophilus* R 26.

2. The method is very specific and allows the determination of a few μg. of deoxyribonucleic acid with a standard deviation of about 10 % even at a low concentration.

The author is indebted to the Rockefeller Foundation for a grant towards laboratory expenses and to Mrs A. Ziesler for valuable technical assistance. The tobacco mosaic virus was a gift from Dr C. A. Knight, University of California.

REFERENCES

Hammarsten, E. (1924). *Biochem. Z.* **144**, 383.
Kalckar, H. M., MacNutt, W. S. & Hoff-Jørgensen, E. (1952). *Biochem. J.* **50**, 397.
Kunitz, M. (1950). *J. gen. Physiol.* **33**, 349.
Rehberg, P. B. (1943). *Acta physiol. scand.* **5**, 277.
Roberts, E. C. & Snell, E. E. (1946). *J. biol. Chem.* **163**, 499.
Schmidt, G. & Thannhauser, S. J. (1943). *J. biol. Chem.* **149**, 369.
Schmidt, G. & Thannhauser, S. J. (1945). *J. biol. Chem.* **161**, 83.
Schneider, W. C. (1945). *J. biol. Chem.* **161**, 293.
Snell, E. E. (1950). *Vitamin Methods*, **1**, 327. New York: Academic Press.

The Enzymic Oxidation and Assay of Adenine

By H. KLENOW
Institute of Cell Physiology, University of Copenhagen

(*Received* 19 *February* 1951)

Booth (1938) showed that adenine is oxidized by xanthine oxidase from milk with the uptake of 1 molecule of oxygen per molecule of adenine oxidized, and that no ammonia is given off. Recently Bendich, Brown, Philips & Thiersch (1950) observed that after oral or intraperitoneal administration of large amounts of adenine to rats, crystals of 2:8-dihydroxyadenine were deposited in the kidneys; they therefore suggested that adenine is oxidized by xanthine oxidase to 2:8-dihydroxyadenine.

The aim of the present work was to characterize the enzymic oxidation product of adenine and to describe a sensitive assay method for this compound.

EXPERIMENTAL

Materials

Adenine (Hoffman-La Roche) was recrystallized as adenine hydrochloride semihydrate.

Xanthine oxidase was prepared from cream according to the method of Ball (1939) with a few modifications (Kalckar, Kjeldgaard & Klenow, 1950*a*).

Characterization of the enzymic oxidation product of adenine

A concentrated solution of adenine (about 10 mg./ml.) in phosphate buffer was incubated under aerobic conditions with catalase and xanthine oxidase (about 5 mg. of protein/ml.); a precipitate gradually formed. The incubation mixture was left at room temperature overnight and the precipitate then washed with water and dissolved in a small amount of 2N-hydrochloric acid. This solution was treated with charcoal and the enzymic reaction product reprecipitated by neutralization with concentrated ammonia. The reprecipitation was repeated three times and the final product was washed and dried over phosphorus pentoxide in vacuum at 100° for 2 hr. This procedure is similar to that used by Bendich *et al.* (1950) for the isolation of 2:8-dihydroxyadenine from rat kidneys. The sample analysed as the semihydrate of 2:8-dihydroxyadenine. (Found: C, 34·8; H, 3·4; N, 40·0. Calc. for $C_5H_5O_2N_5 \cdot \frac{1}{2}H_2O$: C, 34·1; H, 3·4; N, 39·8 %.) After reprecipitation, washing once more, and drying at 140° for 2 hr. the analysis was N, 39·9 %.

The extinction curve of the sample (see Fig. 1) was measured in the Beckman spectrophotometer together with that of the 2:8-dihydroxyadenine isolated from rat kidney (kindly furnished by Dr G. B. Brown, Sloan-Kettering Institute). The measurements were performed with 2 μg./ml. in 0·067 M-phosphate buffer pH 7·0.

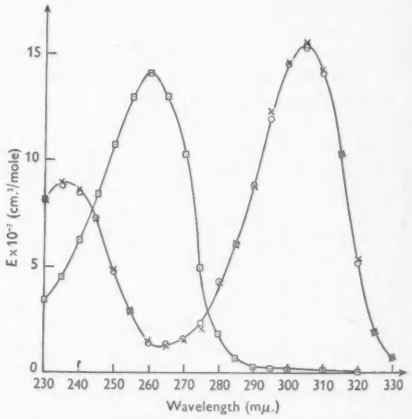


Fig. 1. Molar extinction curves of adenine (□), of the enzymic oxidation product (×) and of 2:8-dihydroxyadenine (○). M/15-Phosphate buffer, pH 7·0.

Addition of crystalline uricase to the oxidation product of adenine as well as to 2:8-dihydroxyadenine did not cause any changes in the absorption at $\lambda = 305$ mμ.

Since apparently the solubility, composition, and the molar absorption curves of the enzymic oxidation product of adenine are the same as those of 2:8-dihydroxyadenine it is reasonable to assume that this compound is formed by the oxidation of adenine by milk xanthine oxidase preparations. In a private communication Dr G. Hitchings has informed us that he also has isolated 2:8-dihydroxyadenine as the oxidation product of adenine after incubation with xanthine oxidase.

Estimation of small amounts of adenine

The assay method is based on enzymic differential spectrophotometry as developed by Kalckar (1947).

Table 1. *Extinction coefficients of adenine and 2:8-dihydroxyadenine*

Compound	Maxima (mμ.)	$E \times 10^{-7}$ (cm.²/g.mol.)	pH	Reference
Adenine	260	1·42	7·0	Gulland & Holiday (1936)
	260	1·41	7·0	Klenow (present paper)
2:8-Dihydroxyadenine	235	0·889	6·8	Bendich *et al.* (1950)
	235	0·880	7·0	Klenow (present paper)
	305	1·54	6·8	Bendich *et al.* (1950)
	305	1·55	7·0	Klenow (present paper)
	Minimum (mμ.)			
2:8-Dihydroxyadenine	263	0·11	7·0	Klenow (present paper)

In Table 1 a list of the absorption maxima and minima and the molecular extinction coefficients of adenine and of 2:8-dihydroxyadenine are given. The maximal differences in absorption and the isobestic points of the two substances at pH 7·0 as derived from Fig. 1 are given in Table 2. The spectral changes at $\lambda = 305$ mμ. found in an experiment starting with different amounts of adenine incubated with xanthine oxidase show (see Table 3) that the method allows estimation down to about 0·3 μg. adenine/ml. with an accuracy of about 10 %.

Table 2. *Density changes at various wavelengths on the oxidation of adenine to 2:8-dihydroxyadenine by milk xanthine oxidase at* pH 7·0

(The values are derived from Fig. 1.)

Wavelength (mμ.)	$\Delta E \times 10^{-7}$ (cm.²/g.mol.)	$\Delta \log I_0/I$ per μg. adenine per ml. $\frac{\Delta E}{1{\cdot}35 \times 10^8}$
243	0	0
260	−1·26	−0·093
277	0	0
305	+1·55	+0·115

Table 3. *Determination of adenine*

(Buffer, M/15-phosphate, pH 7·0; enzyme, xanthine oxidase, 0·5 mg. of protein/ml. mixture.)

Concentration of adenine (μg./ml.)	$\Delta \log I_0/I$ at $\lambda = 305$ mμ. Observed	Calculated from Table 2
0·0163	+0·070	+0·073
0·125	+0·146	+0·144
0·187	+0·197	+0·215

Routine determinations should be carried out at $\lambda = 305$ mμ., not only because this gives the greatest sensitivity, but also because the optical density of most proteins and of other compounds usually present in biological filtrates is low at this wavelength.

The extinction of 2:8-dihydroxyadenine at the maximum at $\lambda = 305$ mμ. changes with the pH of the solution, whereas adenine has no detectable absorption at any pH at this wavelength. Since this causes a considerable difference of the ΔE (change in extinction) per unit of adenine oxidized at different pH, as appears from Table 4, the pH of the assay solution should be well controlled. Since 2:8-dihydroxyadenine is a very insoluble substance (2 μg./ml. of water (Bendich *et al.* 1950)), the buffered solutions for the assay should not contain more than about 2 μg. adenine/ml., as a higher concentration may result in a precipitation of the reaction product.

Table 4. *Molar extinction coefficient of 2:8-dihydroxyadenine at $\lambda = 305$ mμ. at different* pH *values*

Buffer	pH	$E \times 10^{-7}$ (cm.²/mole)	$\Delta \log I_0/I$ per μg. adenine per ml. $\frac{E}{1{\cdot}35 \times 10^8}$
M/15-Phosphate	6·4	1·48	0·110
	7·0	1·55	0·115
	7·4	1·55	0·115
	7·7	1·62	0·120
M/10-Pyrophosphate	7·9	1·69	0·125
	8·4	1·76	0·130
	9·0	1·80	0·133

As mentioned later, the reaction taking place under the conditions usually used for analysis is mainly a first-order reaction. The end point for the reaction, therefore, is not well defined unless rather large amounts of xanthine oxidase are used (for an enzyme preparation with the activity mentioned below about 0·5 mg. of protein/μg. of adenine should be used).

Some properties of the enzymic reaction

The catalytic activity of xanthine oxidase towards adenine is not very high. For the preparation used the initial rate measured as mentioned later corresponds to the oxidation of about 6×10^{-7} g.mol. of adenine/mg. protein/hr. (for hypoxanthine measured under the same conditions according to the method of Kalckar (1947) the figure is about 2×10^{-5} g.mol./mg. protein/hr.). In an assay method the reaction should go to completion at least within

1–2 hr. and a fairly large amount of enzyme, therefore, has to be used. In this case, however, the enzyme protein apparently is present in such a high concentration that, under the experimental conditions, it is no longer saturated with substrate, since the reaction appears to be mainly of the first order (see Fig. 2), whereas most other reactions by xanthine oxidase appear to be mainly of the zero order.

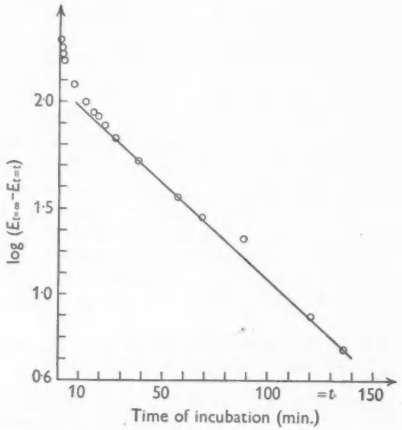


Fig. 2. Enzymic oxidation of adenine. M/15-Phosphate buffer containing about 1·9 μg. adenine and about 0·5 mg. protein/ml.

For the determination of the pH optimum of the reaction it is not sufficient to compare the initial ΔE per minute at different pH values, since the ΔE per minute is dependent not only upon the reaction rate but also upon the total ΔE at a given pH. The pH optimum for the reaction is found, therefore, by comparison of $\frac{\text{initial } \Delta E/\text{minute}}{\text{total } \Delta E}$ at different pH values. The pH optimum was found to be located at about pH 7.

As appears from Fig. 3 the reaction was temporarily inhibited by the presence of 2-amino-4-hydroxy-6-formylpteridine. Thus, the initial reaction rate was only about 25 % of the original in the presence of about 2×10^{-10} g.mol. inhibitor/ml. when the reaction mixture contained about 2×10^{-8} g.mol. adenine/ml. The fact that the inhibition is only temporary is probably due to the enzymic oxidation of the inhibitor (Klenow, 1951). Adenine itself, however, appears to inhibit the activity of xanthine oxidase towards hypoxanthine. Thus, using the method of Kalckar (1947), it was found that in the presence of 7×10^{-8} g.mol. adenine/ml. 0·2 M-phosphate buffer, pH 7·0, containing xanthine oxidase (0·05 mg. protein/ml.) and 7×10^{-8} g.mol. of hypoxanthine/ml. the oxidation rate of hypoxanthine was reduced to about 40 % of the original.

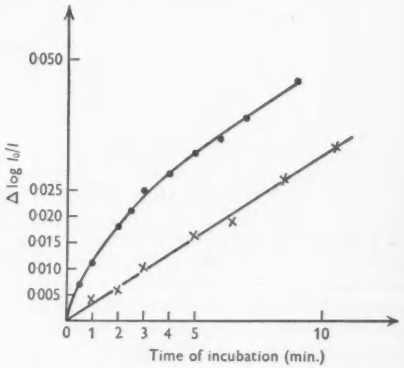


Fig. 3. Inhibition of the enzymic oxidation of adenine by 2-amino-4-hydroxy-6-formylpteridine. ●, 2×10^{-8} Mole of adenine/ml. of M/15-phosphate buffer pH 7·0; ×, the same with 2×10^{-10} mole of inhibitor added/ml. of mixture. Xanthine oxidase (about 0·2 mg. of protein/ml. of mixture) added at zero time.

Procedure for the assay of adenine

The solution containing adenine is buffered with phosphate buffer and the final solution should contain 1–2 μg. adenine/ml. and 0·1–0·2 g.mol. phosphate/ml. The pH of the solution is measured and should be within the range of 7·0–7·5. This solution (3 ml.) is pipetted into a Beckman cuvette. To another cuvette 3·0 ml. phosphate buffer pH 7·0–7·5 and 30 μl. of the xanthine oxidase preparation (containing about 50 mg. protein/ml.) are added. The density at $\lambda = 305$ mμ. of the two solutions is measured; the sum of the densities is called E_{initial}. The reaction is started by addition of 30 μl. of xanthine oxidase to the cuvette containing the adenine solution and is followed at $\lambda = 305$ mμ. until the density does not change for about half an hour; this density is called E_{final}. Then

$$\Delta \log I_0/I = \Delta E = E_{\text{final}} - E_{\text{initial}}$$

and the concentration of adenine is calculated from Table 4.

DISCUSSION

From the experiments mentioned above it is not possible to state if the enzyme oxidizing adenine is identical with the xanthine oxidase from milk. Adenine, however, is probably adsorbed to xanthine oxidase since it turns out to be an inhibitor of this enzyme. In addition the enzymic oxidation of adenine is, like xanthopterin and some aldehydes, inhibited by 2-amino-4-hydroxy-6-formylpteridine (Kalckar, Kjeldgaard & Klenow, 1950*b*). It therefore seems probable that the enzyme oxidizing

adenine is at least of the same type as the enzymes (or enzyme) that oxidize the above-mentioned group of substrates.

SUMMARY

1. The enzymic oxidation product of adenine by milk xanthine oxidase has been identified as 2:8-dihydroxyadenine.

2. An enzymic differential spectrophotometric method for the determination of adenine is described.

This work was generously supported by grants from The Carlsberg Foundation, The Rockefeller Foundation and The Lederle Laboratories Division, American Cyanamid Company.

REFERENCES

Ball, E. G. (1939). *J. biol. Chem.* **128**, 51.
Bendich, A., Brown, G. B., Philips, F. S. & Thiersch, J. B. (1950). *J. biol. Chem.* **183**, 367.
Booth, V. H. (1938). *Biochem. J.* **32**, 494.
Gulland, J. M. & Holiday, E. R. (1936). *J. chem. Soc.* p. 765.
Kalckar, H. M. (1947). *J. biol. Chem.* **167**, 429.
Kalckar, H. M., Kjeldgaard, N. O. & Klenow, H. (1950*a*). *Biochim. Biophys. Acta*, **5**, 575.
Kalckar, H. M., Kjeldgaard, N. O. & Klenow, H. (1950*b*). *Biochim. Biophys. Acta*, **5**, 585.
Klenow, H. (1951). *Math.-fys. Medd.* (in the Press).

Studies Involving Enzymic Phosphorylation

1. THE HEXOKINASE ACTIVITY OF RAT TISSUES

By C. LONG
Department of Biological Chemistry, University of Aberdeen

(*Received* 7 *June* 1951)

Colowick & Kalckar (1941, 1943) have demonstrated that the enzyme hexokinase catalyses the transfer of the terminal phosphate group of adenosinetriphosphate (ATP) to glucose or fructose, yielding the corresponding hexose-6 phosphate and adenosinediphosphate (ADP). Mannose (Berger, Slein, Colowick & Cori, 1946) and glucosamine (Harpur & Quastel, 1949) have also been found to serve as substrates. Mg^{++} is required as a cofactor in these phosphorylations.

The literature concerning the hexokinase activity of animal tissues is extremely fragmentary. Values for homogenates of rat brain and liver have been reported by Utter (1950) and by Vestling, Mylroie, Irish & Grant (1950), respectively. The published figures for rat kidney (Stadie & Haugaard, 1949) and skeletal muscle (Colowick, Cori & Slein, 1947; Stadie & Haugaard, 1949), however, refer to extracts only and were obtained in attempts to discover whether or not the hexokinase activity of animal tissues could be influenced by the *in vitro* addition of hormonal extracts; the lack of agreement in this latter field could conceivably have been due to variations in the experimental conditions adopted by the different workers. Partly in view of this possibility and partly because of the intrinsic value of such information, it seemed desirable to establish the optimal conditions for determining the hexokinase activity of animal tissues and to measure quantitatively the maximal glucose-phosphorylating capacity of each tissue.

In the work to be described, rat-tissue homogenates have been studied and the requirements for maximal activity have been found to be remarkably similar in all tissues. Brain was found to exhibit the greatest hexokinase activity while liver was least active. Other tissues varied over a fourfold intermediate range of activity.

A secondary aim of the present work was to ascertain whether any obvious correlation existed between the hexokinase activity of a tissue and its known function in the economy of the whole animal. As a result of the many enzyme assays performed, no close correspondence could be found for kidney or intestine, in spite of the fact that the phosphorylation of carbohydrate is generally regarded as occupying a special place in the metabolism of these tissues.

EXPERIMENTAL

Materials

Adenosinetriphosphate. ATP was prepared from rabbit muscle as the dibarium salt ($C_{10}H_{12}O_{13}N_5P_3Ba_2.4H_2O$) according to the procedure of Dounce, Rothstein, Beyer, Meier & Freer (1948). Analysis of freshly prepared material: total P, 10·4% (calc. 10·9%); P hydrolysable by N-H_2SO_4 in 10 min. at 100°, 7·3% (calc. 7·3%); hydrolysable P as fraction of total P, 0·70 (calc. 0·67); total N (Kjeldahl),

8·2% (calc. 8·2%); the product was completely free from inorganic orthophosphate. It was stored at −20°; Ba was removed by means of a slight excess of K_2SO_4 to give a 0·05M-stock solution of potassium ATP, which was kept in the refrigerator and freshly prepared about once each month.

Other compounds used were of analytical reagent grade or best available quality and were obtained commercially. Media for the enzymic incubations were prepared in glass-distilled water.

Animals. Hooded rats of the Rowett Institute strain have been used throughout. They were mostly taken from the stock colony maintained by the Physiology Department of this University, but a few were obtained directly from the Rowett Institute, Bucksburn, Aberdeenshire; from the time of weaning they had been fed *ad lib.* a stock diet of rat cake (purchased from the North-Eastern Agricultural Co-operative Society Ltd.). The rats were not fasted except when liver was being assayed; in these cases the animals were deprived of food but not water for 43 hr. in order to reduce the liver glycogen content. Fasting was found to have no effect on the hexokinase activity of other tissues. Male rats, about 3 months old, average weight 300 g., were used, female rats being employed only when uterus was one of the tissues being studied. As there were no apparent sex differences between the hexokinase activities of other tissues, the results obtained on male and female rats have been pooled.

Methods

Animal tissues. The rats were killed by decapitation and exsanguination; the tissues were immediately dissected out and immersed in ice water. After about 2 min., the excess fluid was removed by blotting lightly with filter paper and a portion of cold moist tissue was weighed and homogenized in ice-cold 0·12M-potassium phosphate buffer, pH 7·8, containing 0·15M-KF (5 ml./g. tissue) so that the homogenate contained 0·10M-phosphate and 0·125M-fluoride.

With stomach, small intestine, caecum or colon the organ was dissected free from the mesentery, slit longitudinally and washed in a large beaker in a stream of running tap water to remove undigested food residues and other unwanted material without detaching the mucosa. The tissue was then blotted between filter papers and weighed. When the mucosa of the small intestine was wanted the clean, blotted intestine was placed on a glass plate and the mucosa scraped away from the muscular layers using a blunt spatula. About 45–50% of the weight of the small intestine was removed in this way and this fraction has been designated 'mucosa'.

Dry weights. After drying overnight at 105°, the following percentage dry weights were found for the rat tissues studied: brain, 22·9; caecum, 27·2; colon, 18·3; heart, 22·4; small intestine, 20·8; kidney, 22·9; liver, 31·4; lung, 18·4; skeletal muscle, 22·2; pancreas, 32·4; spleen, 23·8; stomach, 24·0; testis, 12·5; uterus, 19·1.

Preparation of homogenates. In addition to an all-glass homogenizer of the Potter & Elvehjem (1936) type, two other methods have been used. With liver, brain, kidney, heart, testis, uterus, pancreas and intestinal mucosa, suitable homogenates could readily be prepared simply by grinding the tissue in an ice-cold mortar with gradual addition of medium; indeed, for these particular tissues this was the method of choice. In every case it was found that similar hexokinase activities were obtained when these two methods were compared, using tissue from the same animal. Whole small intestine and intestinal muscle, were usually homogenized in an apparatus of the type described by Folley & Watson (1948); this technique also gave values for hexokinase activity similar to those obtained with a Potter & Elvehjem homogenizer. The homogenates were not filtered through gauze for they could readily be pipetted by means of an ordinary 1 ml. graduated pipette with an enlarged tip.

The enzymic phosphorylation of glucose. Solutions containing appropriate amounts of glucose, potassium ATP, $MgCl_2$ and KCl were pipetted into 15 ml. centrifuge tubes, made up to 0·3 ml. with glass-distilled water, and immersed in a Warburg bath at 30°. The homogenate in phosphate-fluoride medium (0·2 ml., containing 33 mg. fresh tissue), prewarmed to 30°, was pipetted in at zero time and the contents gently mixed. After incubation, usually not longer than 10 min., 0·5 ml. 0·3N-$Ba(OH)_2$ was added in order to arrest enzyme activity. After removal from the bath, 5% (w/v) $ZnSO_4$ (0·5 ml.) was added to precipitate the proteins and adsorb the glucose-6-phosphate and other phosphate esters (Somogyi, 1945). Exactly 3·5 ml. water was then added and the contents of the tube thoroughly shaken, centrifuged for about 5 min. at 2000 rev./min. and the almost clear supernatant filtered through a 7 cm. Whatman no. 42 filter paper. 1 ml. of the filtrate was taken for glucose determination according to Nelson (1944), the final colour comparisons being made in a photoelectric colorimeter (Evans Electroselenium Ltd.). While the incubation was proceeding, 0·3 ml. of a standard glucose solution and a similar volume of water were separately treated first with 0·3N-$Ba(OH)_2$ (0·5 ml.) and then with homogenate (0·2 ml.) followed by 5% $ZnSO_4$ (0·5 ml.) and carried through the same procedure except that the incubation was omitted. The amounts of glucose present in the various tubes were calculated from their colorimeter readings together with those of the standard glucose and blank.

RESULTS

Determination of optimal conditions for glucose phosphorylation

General considerations. Glucose was chosen as substrate rather than fructose on account of its much greater affinity for the enzyme, the Michaelis constants for glucose and fructose being $1·45 \times 10^{-4}$ and $1·3 \times 10^{-3}$M, respectively (Wiebelhaus & Lardy, 1949; Slein, Cori & Cori, 1950). Moreover, certain tissues, such as liver and muscle, contain both hexokinase and a specific fructokinase (Slein *et al.* 1950; Vestling *et al.* 1950); thus, results obtained with fructose would be due to the combined effect of both enzymes in these tissues. Although mannose (Michaelis constant, $1·0 \times 10^{-4}$M; Slein *et al.* 1950) is somewhat superior to glucose in its affinity for hexokinase, the maximal rate of mannose phosphorylation is only about 30–40% of that found for glucose (Berger *et al.* 1946).

Earlier workers have usually determined hexokinase activity either by following manometrically the rate of formation of acid groups or by measuring the rate of disappearance of easily hydrolysable (10 min. in N acid at 100°) P. With crude tissue homogenates, however, these methods are certain to give erroneously high values, because the activities of phosphofructokinase, adenosinetriphosphatase and other phosphatases will contribute to the

formation of acid groups, while the first two enzymes will also cause increased loss of easily hydrolysable P. The rate of glucose disappearance has therefore been employed for the determination of hexokinase activity. Broh-Kahn & Mirsky (1948) have argued along similar lines.

Homogenates have been preferred to extracts in this study, for it has been found that a variable fraction of the hexokinase activity of a homogenate passes into the extract. Thus, while 100% of the activity of a stomach homogenate was present in the supernatant after centrifuging at 9000 *g* (angle centrifuge), in two experiments with kidney the corresponding figures were only 14 and 54%; intestinal mucosa gave a value of 57%. Hence, the activity of an extract depends on the nature of the tissue studied and probably also on the thoroughness of centrifugation. In preliminary experiments, the enzymic reaction was carried out under N_2 or a 95% N_2–5% CO_2 mixture, but similar rates of glucose utilization were observed when the incubations were conducted in air. It was also found to be unnecessary to shake the tubes during the incubation.

In the standard test system, 0·2 ml. homogenate, equivalent to 33 mg. wet weight of tissue, was routinely used in a reaction mixture of total volume 0·5 ml. at 30° with a medium of the following final composition, which was found to be optimal: glucose (0·0012 or 0·0024 M), potassium ATP (0·005 M), $MgCl_2$ (0·005 M), KF (0·05 M), potassium phosphate buffer, pH 7·8 (0·04 M) and KCl (0·042 M). Control experiments were always carried out using the complete system without ATP.

Temperature. A few additional experiments were carried out at higher temperatures. The Q_{10} values obtained for homogenates of rat brain, kidney and intestinal mucosa in the range 30–40° were 1·60, 1·47 and 1·52, respectively (average, 1·53).

Cation composition of the medium. Wiebelhaus & Lardy (1949) have shown that the hexokinase activity of an ox-brain extract is somewhat inhibited by an excess of Na^+. In an experiment using rat intestinal mucosa homogenate, it has been confirmed that Na^+ exerts an inhibitory effect on the rate of glucose phosphorylation which is just detectable (Table 1). In view of this finding, an all potassium medium has been used in all subsequent experiments reported in this paper.

Table 1. *Dependence of hexokinase activity on cation composition of reaction medium*

(Rat intestinal mucosa. 0·2 ml. homogenate (≡33 mg. moist tissue) in total volume 0·5 ml. Final concentration of added glucose, 0·0024 M; concentrations of ATP, $MgCl_2$, KF and potassium phosphate, pH 7·8, as for standard test system. Amounts of NaCl and KCl varied to give final concentrations of Na^+ and K^+ as shown. 4 min. incubation at 30°.)

Final concn. (M)		Glucose utilized
Na^+	K^+	(μg.)
0·022	0·165	69
0·043	0·144	69
0·064	0·123	61

Effect of pH *value of the medium.* Three types of experiment have been carried out to test the effect of pH variations on the hexokinase activity: (*a*) tissue homogenates prepared in unbuffered 0·125 M-KF (final concentration) were pipetted into reaction media containing potassium phosphate buffer at various pH values together with all other components of the complete system. (*b*) Weighed portions of tissue were separately homogenized in phosphate-fluoride media of different pH value and the resulting homogenates were then added to the other components of the complete system. (*c*) Tissue homogenates prepared in a phosphate-fluoride medium of pH 7·8 were added to tubes containing the other components of the test system which had earlier been treated with accurately measured amounts of 0·025 N-KOH or 0·125 N-HCl to give known final pH values. In all cases pH values were determined on reaction mixtures containing homogenate, using a pH meter with glass electrode.

The results of these experiments are shown in Table 2. While the hexokinase activity of tissue homogenates did not appear to be highly sensitive to pH changes, optimum values were usually obtained in the pH range, 7·5–8·0. In view of these findings, homogenates have been prepared in phosphate buffer, pH 7·8, for routine determinations of hexokinase activity.

Table 2. *Hexokinase activity of rat-tissue homogenates at varying* pH *values*

(Methods (*a*), (*b*) and (*c*) described in text. Final concentrations of ATP, $MgCl_2$, KCl, KF and potassium phosphate as for standard test system. In Exp. 3, final glucose concentration, 0·0024 M; 10 min. duration. In all other experiments, final glucose concentration, 0·0012 M; 8 min. duration.)

Exp. no.	Method	Rat tissue	Final pH value	Glucose utilized (μg.)
1	(*a*)	Kidney	6·20	56
			6·60	52
			7·09	94
			7·57	117
			7·90	115
			8·15	104
2	(*a*)	Intestinal mucosa	6·38	107
			6·81	107
			7·31	108
			7·80	134
			8·15	95
			8·56	89
3	(*b*)	Intestinal mucosa	6·38	88
			7·80	92
			8·56	72
4	(*b*)	Kidney	7·80	56
			8·55	57
5	(*c*)	Kidney	5·71	59
			6·38	64
			7·49	54
			7·87	55
			8·19	58

Effect of glucose concentration. In rat intestinal mucosa and stomach homogenates the rate of glucose utilization hardly varies with glucose concentration over the range studied. This effect is shown in Table 3. In their study of the hexokinase activity of ox-brain extracts, Wiebelhaus & Lardy (1949) experienced a similar behaviour, for on raising the glucose concentration from 0·0005 to 0·005 M, the rate of phosphorylation was not increased by more than 5%.

Table 3. *Hexokinase activity at varying glucose concentrations*

(Standard test system, except for varying glucose concentration.)

Initial glucose concentration (M)	Glucose utilized (μg.) Intestinal mucosa (4 min.)	Stomach (5 min.)
0·0012	74	89
0·0024	77	—
0·0036	80	97

Effect of ATP concentration. With glucose as substrate, Slein *et al.* (1950) determined the affinity of crystalline yeast hexokinase for ATP and reported a Michaelis constant of $9{\cdot}5 \times 10^{-5}$ M. With ox-brain extracts, Wiebelhaus & Lardy (1949) found that an initial ATP concentration of 0·002 M was sufficient for maximal activity when fairly small amounts of glucose were being utilized. In the present work (Table 4) a somewhat higher concentration of ATP was

Table 4. *Hexokinase activity at varying ATP concentrations*

(Standard test system, except for variations in ATP concentration.)

	Glucose utilized (μg.) Intestinal mucosa (8 min.)	Heart (6 min.)	Kidney (8 min.)
Initial glucose (M)	0·0024	0·0012	0·0012
ATP concentration (M)			
0·0000	−4	20	2
0·0013	60	48	34
0·0025	97	60	45
0·0038	118	84	66
0·0050	139	86	71
0·0063	138	—	71

required to give the maximal rate of glucose utilization, presumably because of the competitive destruction of ATP by crude homogenates (Meyerhof & Geliazkowa, 1947). Maximal hexokinase activity was always obtained when the concentration of ATP reached 0·005 M. In most tissues there was a slight formation of reducing substance, estimated as glucose, during the course of the incubation in the absence of ATP. Where this effect was observed, the glucose utilization has been taken to be the difference between the amounts of glucose present in the complete system and in the same without ATP at the end of the incubation period. An effect of similar nature has also been noted by Stadie, Haugaard & Hills (1950) using an extract of cat skeletal muscle.

Effect of Mg^{++} *concentration.* Under conditions which are otherwise optimal, but without added Mg^{++}, the rate of glucose utilization by tissue homogenates was very low; maximal activity appeared to be obtained when the concentration of added $MgCl_2$ reached 0·005 M (Table 5).

The actual concentration of Mg^{++} in these unsupplemented tissue homogenates has not been determined. However, Leut'skii (1946) has reported normal values of 101, 69 and 78 mg. Mg/100 g. for rat muscle, brain and liver, respectively. Assuming an average value of 80 mg./100 g. tissue, the unsupplemented reaction mixtures, as prepared in the present work, should contain about 0·002 M-Mg^{++}. Thus it is quite likely that Mg^{++} is essential for hexokinase activity.

Table 5. *Effect of* Mg^{++} *concentration on hexokinase activity*

(Standard test system, except for Mg^{++} concentration.)

	Glucose utilized (μg.) Intestinal mucosa (8 min.)	Brain (5 min.)	Testis (8 min.)
Initial glucose (M)	0·0024	0·0024	0·0012
Mg^{++} concentration (M)			
0·0000	48	38	23
0·0015	92	107	63
0·0030	103	116	78
0·0050	110	127	84
0·0075	101	135	75

Effect of fluoride concentration. In most animal tissue preparations, the hydrolysis of glucose-6-phosphate at pH 7·8 is probably brought about by non-specific alkaline phosphatase. In liver, kidney and possibly intestinal mucosa, a specific glucose-6-phosphatase is also active (Broh-Kahn, Mirsky, Perisutti & Brand, 1948; Swanson, 1950; Duve, Berthet, Hers & Dupret, 1950; Hers & Duve, 1950). In general, fluoride ions are known to inhibit phosphate splitting enzymes, and in the present work the rate of disappearance of glucose from the reaction mixture has often been found to be raised appreciably by increasing concentrations of KF (Table 6); where an effect is seen, it appears to be maximal at about 0·05 M-KF. The presence of fluoride in the medium has the additional advantage of inhibiting ATPase to a considerable extent.

Table 6. *Effect of fluoride concentration on hexokinase activity*

(Standard test system, except for variable KF concentration.)

	Glucose utilized (μg.) Kidney (8 min.)	Pancreas (6·5 min.)	Intestinal mucosa (10 min.)
Initial glucose (M)	0·0012	0·0012	0·0024
KF concentration (M)			
0·000	30	38	72
0·015	60	37	114
0·030	66	37	114
0·050	71	35	112
0·075	57	40	107

In the case of kidney (Table 6), the need for fluoride is evident and has consistently been found. In pancreas, however, fluoride has no noticeable effect and a similar result has been obtained for lung. With intestinal mucosa the response was variable; in the experiment shown (Table 6), maximal hexokinase activity was seen in the presence of 0·015 M-KF, higher concentrations producing no further activation. In another experiment (not quoted) fluoride was without effect. This latter result is surprising, especially in view of the finding by Hers & Duve (1950) that glucose-6-phosphate is optimally hydrolysed by intestinal mucosa at pH 8·0.

That the fluoride ion, up to a concentration of 0·1 M, has no inhibitory effect on hexokinase has been shown for the crystalline yeast enzyme by Berger *et al.* (1946). A similar result is that shown for pancreatic tissue (Table 6), the hexokinase activity of which is not inhibited by KF up to 0·075 M. In view of the hydrolysis of glucose-6-phosphate which would normally take place in homogenates of tissues such as kidney, routine determinations of hexokinase activity have been carried out in the presence of 0·05 M-KF. In order to avoid the precipitation caused by the interaction of fluoride, ATP and Mg^{++} in concentrated solution, the tissue was homogenized in a fluoride-phosphate medium and added to the rest of the reaction mixture containing ATP and Mg^{++}. In experiments on guinea pig brain, Case & McIlwain (1951) have recommended that the homogenization of the tissue be carried out in a fluoride-containing medium in order to reduce the phosphatase activity.

Since any method of hexokinase assay, based on the rate of disappearance of glucose, requires that enzymic hydrolysis of the phosphorylated product, glucose-6-phosphate, should not take place, separate experiments have been carried out to determine whether 0·05 M-KF would quantitatively inhibit the hydrolysis of added glucose-6-phosphate. It has been found in the case of homogenates of every tissue studied, that this fluoride concentration completely prevented the formation of reducing substances from hexosemonophosphate (equilibrium mixture of glucose-6-phosphate and fructose-6-phosphate) at an initial concentration of 0·0005 M, i.e. at a concentration equivalent to that produced by phosphorylation of more than 80% of the glucose used in the standard test system.

Effect of enzyme concentration. In those tissues which were examined, the rate of glucose utilization was directly proportional to the volume of homogenate taken, within the limits of experimental error, other components of the medium being maintained constant (Table 7). The absence of any falling off in the rate of reaction either at lower or higher concentrations of homogenate may be taken as evidence that there are no other co-factors to be considered and that the known co-factors are present in optimal amount.

Table 7. *Dependence of rate of glucose utilization on volume of homogenate taken*

(Standard test system, except for variation in amount of tissue taken. 1 ml. homogenate equivalent to 167 mg. fresh tissue.)

	Glucose utilization (μg.)		
	Testis (10 min.)	Brain (6 min.)	Intestinal mucosa (7 min.)
Initial glucose (M)	0·0012	0·0024	0·0012
Homogenate (ml.)			
0·05	19	38	27
0·10	43	73	52
0·20	72	167	88

Effect of duration of incubation. Provided not more than about 80% of the initial glucose was used up, the following rat tissues showed strict proportionality between duration of incubation and extent of glucose utilization: brain, caecum, colon, heart, small intestine, kidney, lung, stomach, testis and uterus; i.e. the reaction was of zero order. In the case of intestinal mucosa, the reaction rate decreased appreciably as the time of incubation was lengthened; in twenty-two experiments, the average quantity of glucose disappearing during a second 4 min. period was only 72% of the amount utilized during the first 4 min. Thus in calculating the hexokinase activity of intestinal mucosa, only the early period of incubation has been considered. With pancreas, a similar but even more pronounced effect was observed and in this case too, only data referring to short periods of incubation have been employed.

With rat skeletal muscle, an opposite effect was seen. During short incubation periods of about 5 min. or less, a negligible amount of glucose was utilized; indeed, the presence of ATP in the medium often appeared to increase the amount of glucose present, as compared with a control without ATP, so that negative rates of glucose utilization were observed under these conditions. With longer periods of incubation, however, a positive and linear rate of reaction was consistently found. For this reason, when calculating the hexokinase activity of skeletal muscle homogenates, it has been necessary to discard the earlier values and use only the figures relating to the longer periods of incubation, e.g. for the period 6–12 min. Spleen behaved in a similar manner. Perhaps the simplest explanation for these observations would be that these tissues contained a hexokinase inhibitor which gradually underwent destruction during the incubation.

Liver presented a special problem owing to its high glycogen content. Fasted rats were necessarily used when assaying this tissue for hexokinase, for otherwise the excessive formation of glucose during the preparation of the homogenate and subsequent incubation flooded the system to such an extent that it was impossible to observe any disappearance of glucose during the test period. Even with liver from fasted rats, there was still sufficient glycogen to cause a net increase in glucose concentration during the first few minutes of incubation. However, it was found that if a preliminary incubation period of 10 min. at 30° was allowed before mixing the homogenate with the other components of the system, a small though measurable amount of glucose was utilized at a rate approximately proportional to the duration of the test.

The hexokinase activity of rat tissues

A summary of the results of individual experiments is recorded in Table 8, in which the tissues

Table 8. *Hexokinase activity of rat tissues*

(Standard test system.)

Tissue	No. of exps.	$-Q_{glucose}$ Average	$-Q_{glucose}$ Range
Brain (whole)	6	27·1	20·3–31·2
Colon	4	17·2	12·6–21·4
Heart	9	14·5	9·2–17·8
Stomach	3	14·5	8·3–20·4
Testis	6	13·7	11·4–18·8
Small intestine	3	11·7	9·3–13·3
Caecum	2	10·7	10·4–10·9
Uterus	4	8·5	6·0–10·9
Spleen	3	8·3	7·3– 9·3
Kidney (whole)	12	7·9	6·3–11·0
Skeletal muscle	8	7·4	1·7–12·0
Pancreas	4	5·6	4·0– 7·4
Lung	3	4·3	2·9– 5·6
Liver	8	1·4	0·2– 2·6

have been arranged in order of decreasing hexokinase activity. The hexokinase activities of the mucosal and muscular portions of small intestine are compared in Table 9. Hexokinase activity is given by the rate of glucose utilization and has been calculated in terms of $-Q_{glucose}$ (μl. glucose disappearing/mg. dry wt. of tissue/hr.).

Table 9. *Hexokinase activities of the mucosal and muscular portions of small intestine*

(Standard test system. In each experiment, the mucosa and muscle were taken from the small intestine of the same animal.)

	$-Q_{glucose}$	
Exp. no.	Mucosa	Muscle
1	14·1	11·8
2	17·6	13·9
3	10·1	9·7
	Average 13·9*	11·8

* Average of eleven experiments, 14·8 (range, 10·1–20·2).

DISCUSSION

Perhaps the most striking fact which emerges from an inspection of the data in Table 8 is the very high hexokinase activity of rat-brain homogenate, which is almost double that of any other tissue, and the very low activity of rat liver. In Table 10 the maximal phosphorylating capacities at 38° of various rat-tissue homogenates have been calculated in terms of mg. glucose/whole tissue/hr. In order to convert from 30 to 38°, a factor of 1·41 has been employed, corresponding to an average Q_{10} value of 1·53 (see p. 409).

Table 10. *Maximal glucose phosphorylating capacities of rat tissues*

(The rates of glucose phosphorylation at 38° have been calculated from the following data: (*a*) $-Q_{glucose}$ at 30° (Table 8); (*b*) average $Q_{10}=1{\cdot}53$; (*c*) moist weight of tissue; and (*d*) percentage dry weight of tissue. See Experimental section, p. 409.)

Tissue	Average wt. of whole moist tissue (g.)	Maximal rate of glucose phosphorylation (mg./whole tissue/hr.)
Brain	1·89	132
Caecum	1·76	57
Colon	1·97	70
Heart	1·00	38
Kidneys	2·27	46
Liver	6·59*	33
Lung	1·58	14
Pancreas	0·92	20
Skeletal muscle	150†	2780
Small intestine	9·12	251
Intestinal mucosa	4·37	152
Spleen	1·98	44
Stomach	1·59	62
Testes	2·28	45
Uterus	0·44	9

* Fasted liver used here; weight of non-fasted liver, approx. 10 g.

† Approximate only, assuming 50% of a 300 g. rat to be muscle.

Brain. The dependence of the *in vivo* respiratory metabolism of brain upon the level of the blood glucose would lead to the expectation of a high hexokinase activity for this tissue. Utter (1950), working with a homogenate of cotton rat brain, obtained a value of 37·8 for $-Q^{O_2}_{glucose}$ at 38°. When the temperature difference is taken into account, the average value of 27·1 at 30° found in the present work is equivalent to 38·2 at 38°, which agrees closely with Utter's (1950) figure. Such a rate of glucose phosphorylation is considerably higher than that required to support the observed rate of respiration of rat brain, for Krebs (1934) has quoted an average $-Q_{O_2}$ value of 10·7 for slices of rat-brain cortex at 38°, a figure which would require a $-Q_{glucose}$ value of only 1·8, assuming complete oxidation of the glucose molecule. Reiner (1947) has also considered this problem, and while he did not determine directly the glucose utilization of rat-brain homogenates, the manometrically determined values of $-Q_{O_2}$ and $Q^{O_2}_{glycolysis}$ indicated that for every molecule of glucose completely oxidized, 12 mol. of glucose were converted into lactic acid. Thus it appeared that the rate of pyruvate oxidation was the limiting factor in the complete oxidation of glucose by rat brain. Under *in vivo* conditions, it is possible that suboptimal concentrations of co-factors and the presence of uninhibited phosphatases would reduce the net rate of glucose phosphorylation considerably. Whatever may be the complete explanation, the results clearly show that the hexokinase activity of the brain is more than sufficient to meet the metabolic requirements of the rat.

Kidney. All previously published data on the hexokinase activity of kidney refer to extracts. Glucose phosphorylation in kidney extracts was first observed by Colowick, Welch & Cori (1940) using the ox, lamb, pig, cat and rabbit, but their results do not indicate which species was used in any given experiment. Later Youngburg (1944), using rabbit-kidney cortex extracts at 39–40°, obtained data from which a $-Q_{glucose}$ value of 4·2 may be calculated. More recently, Stadie & Haugaard (1949) have employed rat-kidney extracts at 30° under conditions rather similar to those used in the present study except for the addition of adrenal cortex extract, which the authors stated to be without effect; their results may be recalculated to give an average $-Q_{glucose}$ value of 2·4. In the present work, the determinations made upon kidney homogenate and extract, already referred to, have indicated that not more

than about 50 % of the hexokinase activity remains in the supernatant, so that the higher average $-Q_{glucose}$ value of 7·9 (Table 8) obtained for kidney homogenate is to be expected.

It seemed of interest to ascertain whether the $-Q_{glucose}$ value observed in this study could be correlated with the performance of rat kidneys under physiological conditions. No data could be found in the literature for the maximal rate of glucose re-absorption from the glomerular filtrate in the rat, but experiments on inulin clearance by Friedman & Livingstone (1942) and Dicker & Heller (1945) have yielded values of $0{\cdot}27 \pm 0{\cdot}06$ and $0{\cdot}35 \pm 0{\cdot}04$ ml./100 g. body weight/min. respectively for the glomerular filtration rates of albino rats. Taking an average figure of 0·30 ml./100 g. body weight/min. together with a mean value of 120 mg. glucose/100 ml. arterial blood found for the rats from our colony (Taylor, 1951), and assuming the glucose concentrations in whole blood and plasma to be equal, it can be calculated that a 300 g. rat must be able to reabsorb not less than 65 mg. glucose/hr. from the glomerular filtrate under normal conditions. This figure is to be compared with the value of 46 mg. which is the maximal amount of glucose which may be phosphorylated per hr. as calculated from the hexokinase activity (Table 10). The difference would be even greater at a blood glucose level corresponding to a renal threshold of about 180 mg. glucose/100 ml. blood. This discrepancy is difficult to resolve unless it be assumed that under hyperglycaemic conditions about half of the glucose of the glomerular filtrate is reabsorbed by passive diffusion. That this may be the true explanation is suggested by the observations of Hemingway (1939) who determined the ratio of the inulin and xylose clearances in the dog and found that about 31 % of the pentose of the glomerular filtrate was reabsorbed, presumably by a passive mechanism.

Intestine. The hexokinase activity of the mucosal portion of rat small intestine appears to be slightly greater than that of the muscular portion (Table 9). Apparently also the glucose phosphorylating capacity of rat colon is higher than that of small intestine (Table 8). Since it has usually been accepted that 'active' absorption of sugars does not take place from the colon, stomach or caecum, the fact that their hexokinase activities are comparable in magnitude with that of intestinal mucosa is somewhat surprising.

The recently published data of Hele (1950) on glucose phosphorylation by rat intestinal mucosa at 30°, when converted into $-Q_{glucose}$ units, yield average values of 23·4 (water homogenates) and 37·5 (magnesium Ringer homogenates). These figures are nearly double those found in the present work.

The diet of the adult hooded rats used in this research normally contains about 12 g. carbohydrate/day. Table 10, however, shows that the mucosa of the small intestine possesses a phosphorylating capacity of only 152 mg. glucose/hr. or 3·65 g./day. In fact, in order to account for the phosphorylation of 12 g. carbohydrate/day, it would be necessary to take into consideration the whole of the alimentary tract. Even if the highest hexokinase activity of Hele (1950) were employed in the calculation, the total absorption of this amount of glucose could not be accounted for by the mucosa alone. In experiments to determine the *in vivo* rate of absorption, Hele (1950) found that 57 mg. glucose were absorbed in 30 min. This would give an absorption rate of only 2·7 g. glucose/day, a value which is probably much too low, for Cori (1925) obtained an average figure of 172 mg. glucose/100 g. body weight/hr., equivalent to 12·4 g./24 hr.

As in the case of kidney, the possibility must be considered that a fraction of the glucose is absorbed from the intestine by passive diffusion.

Skeletal muscle. The hexokinase activity of homogenates is probably significantly higher than of extracts of skeletal muscle. The $-Q_{glucose}$ value of 7·4 (Table 8), found for homogenates, is to be compared with 2·8 and 2·6 calculated from the data of Colowick *et al.* (1947) and Stadie & Haugaard (1949), respectively, for extracts.

The anomalous behaviour of rat-skeletal muscle homogenates during short incubation periods has already been referred to. This initial period of inhibition superficially resembles that observed by Colowick *et al.* (1947) with muscle extracts from alloxan-diabetic rats, and in further experiments has been found to be relieved by insulin. The fact that a spleen homogenate behaved similarly may be related to the observation of Broh-Kahn & Mirsky (1947) that spleen extracts inhibited the hexokinase activity of muscle extracts in the same manner as anterior pituitary extract.

Liver. The low hexokinase activity of rat-liver homogenates was not unexpected, although Slein *et al.* (1950), by contrast, consistently observed fairly high rates of glucose phosphorylation with homogenates from the livers of their strain of rats. In a recent paper, Vestling *et al.* (1950), on the other hand, found that their rat-liver homogenates readily catalysed the phosphorylation of fructose by ATP, but had little action on glucose. This observation was not caused by rapid dephosphorylation of the glucose-6-phosphate formed by glucose-6-phosphatase for the liver homogenate had no effect on the activity of a brain-hexokinase preparation.

These latter results are also in agreement with the findings of Chernick & Chaikoff (1951) who incubated rat-liver slices in a medium containing

uniformly labelled [^{14}C]-glucose and determined the amount of ^{14}C incorporated into the fatty acids and respiratory carbon dioxide. If it be assumed that the formation of these two products accounts for the whole of the glucose utilized, then a $-Q_{glucose}$ value of only 0·8 can be calculated from their data. The possibility must be considered, however, that other reactions involving C_2 fragments, derived from [^{14}C]-glucose, are also taking place, e.g. acetylation, formation of ketone bodies, etc. A recent paper by Sacks (1951) may also be taken as support for the present findings. This author studied the relative specific activity: time relationships of the acid-soluble phosphate esters of rat liver after subcutaneous injection of inorganic ^{32}P, and presented evidence against the idea that the labile P of ATP could be the precursor of the stable P of glucose-6-phosphate.

Table 10 shows that the maximal glucose phosphorylating capacity of rat liver is only about 33 mg./hr. This value is considerably lower than many of the published figures for the rate of liver-glycogen synthesis in the intact rat. Thus Treadwell, Tidwell & Grafa (1943) on feeding glucose to rats previously fasted for 24 hr. observed an average rate of liver-glycogen synthesis of 74 mg./hr., corresponding to a glucose equivalent of 82 mg./hr.

Parallel with these observations is the well known difficulty of demonstrating the conversion of glucose into glycogen in the presence of liver slices from fasted rats or rabbits (Cross & Holmes, 1937; Hastings & Buchanan, 1942; Saxton & Miller, 1944). Similarly, glucose does not raise the level of respiration when added to liver slices from fasted rats, although such tissue preparations respond well to the addition of sodium pyruvate.

The simplest explanation of these findings would be that the liver glycogen is normally formed by glyconeogenesis from C_3 fragments produced from glucose in the peripheral tissues. Such a mechanism would not involve a liver hexokinase.

SUMMARY

1. Homogenates of several rat tissues have been assayed for hexokinase activity, using the rate of glucose disappearance in the presence of adenosinetriphosphate as a measure of activity.

2. Optimal rates of glucose utilization were obtained when the homogenates were prepared in a potassium phosphate buffer, pH 7·8, containing potassium fluoride, and the incubation medium contained the following components (final concentrations in brackets): glucose (0·0012–0·0024M); potassium adenosinetriphosphate (0·005M); magnesium chloride (0·005M); potassium fluoride (0·05M); potassium phosphate buffer, pH 7·8 (0·04M); potassium chloride (0·042M). The incubation was conducted in air at 30° and varied from 2 to 16 min. duration.

3. Under the above conditions, the amount of glucose utilized was proportional to the volume of homogenate used and in most cases increased linearly with the duration of incubation until at least 80% of the glucose had disappeared.

4. Results, expressed as $-Q_{glucose}$ (μl. glucose disappearing/mg. dry wt. of tissue/hr.) showed that brain (27·1) was the most active tissue and liver (1·4) the least active.

5. A number of tissues have been considered in the light of the relationship between their hexokinase activities and their physiological functions.

It is a pleasure to thank the following: Dr J. Laguna, who collaborated in the preparation of the sample of barium adenosinetriphosphate used in this work and who carried out the analysis of the specimen; Miss I. Creighton, for help with the rats; and Mr W. R. Ferrier, for skilled technical assistance. It is also desired to thank the Medical Research Council for an Apparatus Grant.

REFERENCES

Berger, L., Slein, M. W., Colowick, S. P. & Cori, C. F. (1946). *J. gen. Physiol.* **29**, 379.

Broh-Kahn, R. H. & Mirsky, I. A. (1947). *Science*, **106**, 148.

Broh-Kahn, R. H. & Mirsky, I. A. (1948). *Proc. Soc. exp. Biol., N.Y.*, **67**, 176.

Broh-Kahn, R. H., Mirsky, I. A., Perisutti, G. & Brand, J. (1948). *Arch. Biochem.* **16**, 87.

Case, E. M. & McIlwain, H. (1951). *Biochem. J.* **48**, 1.

Chernick, S. S. & Chaikoff, I. L. (1951). *J. biol. Chem.* **188**, 389.

Colowick, S. P., Cori, G. T. & Slein, M. W. (1947). *J. biol. Chem.* **168**, 583.

Colowick, S. P. & Kalckar, H. M. (1941). *J. biol. Chem.* **137**, 789.

Colowick, S. P. & Kalckar, H. M. (1943). *J. biol. Chem.* **148**, 117.

Colowick, S. P., Welch, M. S. & Cori, C. F. (1940). *J. biol. Chem.* **133**, 359.

Cori, C. F. (1925). *J. biol. Chem.* **66**, 691.

Cross, M. C. A. & Holmes, E. G. (1937). *Brit. J. exp. Path.* **18**, 370.

Dicker, S. E. & Heller, H. (1945). *J. Physiol.* **104**, 353.

Dounce, A. L., Rothstein, A., Beyer, G. T., Meier, R. & Freer, R. M. (1948). *J. biol. Chem.* **174**, 361.

Duve, C. de, Berthet, J., Hers, H. G. & Dupret, L. (1950). *Bull. Soc. Chim. biol., Paris*, **31**, 1242.

Folley, S. J. & Watson, S. C. (1948). *Biochem. J.* **42**, 204.

Friedman, S. M. & Livingstone, C. A. (1942). *Amer. J. Physiol.* **137**, 564.

Harpur, R. P. & Quastel, J. H. (1949). *Nature, Lond.*, **164**, 693.

Hastings, A. B. & Buchanan, J. M. (1942). *Proc. nat. Acad. Sci., Wash.*, **28**, 478.
Hele, M. P. (1950). *Nature, Lond.*, **166**, 786.
Hemingway, A. (1939). *J. Physiol.* **95**, 3*P*.
Hers, H. G. & Duve, C. de (1950). *Bull. Soc. Chim. biol., Paris*, **32**, 20.
Krebs, H. A. (1934). *Tabul. biol. Berl.* **9**, 209.
Leut'skii, K. H. (1946). *Ukr. Biochem. J.* **18**, 87. Cited by *Chem. Abstr.* (1947), **41**, 6947.
Meyerhof, O. & Geliazkowa, N. (1947). *Arch. Biochem.* **12**, 405.
Nelson, N. (1944). *J. biol. Chem.* **153**, 375.
Potter, V. R. & Elvejhem, C. A. (1936). *J. biol. Chem.* **114**, 495.
Reiner, J. M. (1947). *Arch. Biochem.* **12**, 327.
Sacks, J. (1951). *Arch. Biochem.* **30**, 423.
Saxton, J. A. & Miller, M. L. (1944). *Arch. Path.* **37**, 34.
Slein, M. W., Cori, G. T. & Cori, C. F. (1950). *J. biol. Chem.* **186**, 763.
Somogyi, M. (1945). *J. biol. Chem.* **160**, 69.
Stadie, W. C. & Haugaard, N. (1949). *J. biol. Chem.* **177**, 311.
Stadie, W. C., Haugaard, N. & Hills, A. G. (1950). *J. biol. Chem.* **184**, 617.
Swanson, M. A. (1950). *J. biol. Chem.* **184**, 647.
Taylor, D. W. (1951). Private communication.
Treadwell, C. R., Tidwell, H. C. & Grafa, B. G. jun. (1943). *J. biol. Chem.* **149**, 209.
Utter, M. F. (1950). *J. biol. Chem.* **185**, 499.
Vestling, C. S., Mylroie, A. K., Irish, U. & Grant, N. H. (1950). *J. biol. Chem.* **185**, 789.
Wiebelhaus, V. D. & Lardy, H. A. (1949). *Arch. Biochem.* **21**, 321.
Youngburg, G. E. (1944). *Arch. Biochem.* **4**, 137.

Blood Clotting: the Function of Electrolytes and of Calcium

By J. E. LOVELOCK and B. M. PORTERFIELD
Medical Research Council, Common Cold Research Unit, Harvard Hospital, Salisbury

(*Received* 10 *May* 1951)

The theory of blood clotting advanced by Morawitz (1904) and Fuld & Spiro (1904) postulated that the presence of free calcium ions was a necessary condition of coagulation. This was based on the evidence of Arthus & Pagés (1890) and Pekelharing (1892) that certain anions, namely, oxalate and citrate, whose calcium salts have low solubility products, inhibit the clotting of blood. Vines (1921), and Stewart & Percival (1928) have commented on the unsatisfactory nature of this evidence, and the recent work of Quick & Stephanini (1948) has cast further doubt on these accepted beliefs. They have suggested that calcium functions in blood clotting, not as a free ion, but in combination with prothrombin as an unionized complex. Also that the activity of citrate is not connected with the low solubility of its calcium salt. If these suggestions are accepted, it is of some interest to consider the alternative theories which can be put forward to explain the anticoagulant activity of oxalate and citrate anions.

(1) Oxalate and citrate ions form complexes with low solubility products with other elements, in particular, iron, cobalt and zinc. If it is assumed that some enzyme incorporating one of these elements is necessary for coagulation, then the anions might lower its activity by removal of an essential element from the enzyme.

(2) The process of coagulation involves the interaction of electrically charged colloidal particles. Ions, particularly multivalent ions, are known to exert an influence on such processes and the activity of oxalate and citrate ions may be due to this effect.

The second theory, namely that ions such as oxalate and citrate might act as anticoagulants by a non-specific salt effect was first proposed by Schmidt (1895). Interest in this possibility was, however, largely dropped when Morawitz (1904) suggested that ionized calcium acted catalytically in the coagulation of blood, and when Sabbatani (1908) explained the action of citrate ions in terms of the low solubility product of its calcium salt.

The only systematic studies of the action of neutral salts on coagulation are those of Glazko & Greenburg (1939), Astrup (1944) and Mommaerts (1945). These authors investigated the anticoagulant activity of anions, including oxalate and citrate ions, on the clotting of purified fibrinogen by thrombin. They showed that in this second phase of blood coagulation, the anticoagulant activity of the anions tested was connected with their valency.

The results reported in this paper were obtained by observing the effect of various anions on the coagulation of whole blood, and by determining the concentration and relative proportions of electrolytes needed to restore the coagulability of salt-free plasma. Many physiological processes are known to be highly sensitive to changes in the concentration and species of ions present in the suspending medium. The results indicate the process of coagulation to be similarly sensitive to disturbances in the electrolyte balance of the blood. In particular they

suggest that coagulation can only occur when the total ionic strength of the blood is within prescribed limits, and only when a certain proportion of the cations present are of a suitable species. They indicate that the anticoagulant activity of oxalate, citrate and fluoride ions is due not only to their ability to depress the calcium ion concentration of blood, but also to their ionic charge; with citrate ions the latter effect predominates, with fluoride ions the former. They also suggest there is no need to ascribe any special function, such as catalytic activity, to the element calcium in the coagulation process, or the formation of defined compounds with the elements of the blood, but instead suggest that calcium functions by maintaining an electrolyte balance suitable for the interaction of the various plasma colloids.

METHODS

Determination of the concentration of anions needed to prevent the coagulation of whole blood. Human blood was collected by venipuncture using a cold dry syringe and transferred immediately to a polyethylene beaker kept at 0°. Portions (0·2 ml.) were transferred by Pasteur pipettes to small glass tubes each containing 0·2 ml. of a solution of the Na or K salt of the test anion. These tubes were kept at 20° and examined after 12 hr. for clotting. The syringe and pipettes were treated before use with dimethyldichlorosilane to render their surfaces inert towards the blood (Jacques, Fidlar, Feldstedt & Macdonald, 1946).

The preparation of calcium-free plasma. The decalcification of blood by cation-exchange resins was first described by Steinberg (1944) and developed into a laboratory method by Stephanini (1948). The method described below is similar to that reported by Stephanini, and we can confirm that the use of cation-exchange resins provides a convenient and effective method of decalcifying blood without otherwise altering its properties.

Human blood was collected by the procedure described earlier, and transferred to a polyethylene flask containing the sodium form of the cation-exchange resin Zeo-Karb 225. For each 10 ml. of blood added 4 g. were used. The mixture of blood and resin was kept at 0° and gently stirred for a period of 15 min. The blood was then separated from the resin by filtration and the plasma from the blood by centrifugation. All glassware used in these operations was treated before use with dimethyldichlorosilane. The plasma produced by this method contained less than 0·2 μg. Ca/ml. and was stored until required in polyethylene flasks at 0°.

The preparation of salt-free plasma. Plasma decalcified by the method just described was dialysed for 24 hr. at 20° against running distilled water to remove salts. It was found necessary to take considerable precautions to prevent the access of Ca to the plasma during dialysis. When the ionic strength of plasma is 0·03 as little as 3 μg. Ca/ml. is sufficient to cause coagulation. The dialysis apparatus was therefore constructed of polyethylene and the cellophan membrane washed thoroughly in N-HCl before use to remove traces of Ca. In earlier experiments in which glass funnels were used to support the dialysis membrane clotting frequently occurred during dialysis. Experiments described later in this paper suggest this was probably due to the liberation of Ca from the glassware.

RESULTS

The inhibition of coagulation by anions. Table 1 shows the concentrations of various anions needed to prevent coagulation. Also shown are the solubility products of the calcium salts of the anions

Table 1. *The anticoagulant activity of anions with reference to their valency and their ability to remove calcium ions from solution*

(Measurements were made of the concentration of salt required to prevent the coagulation of human blood in Pyrex glass tubes at 20°.)

			Ca salt	
Anion	Valency	Concn. required to prevent coagulation (mM)	Solubility product	Calcium ion concn. of saturated aqueous solution (mM)
Azide	1	300	Soluble	—
Chloride		300	Soluble	—
Fluoride		80	$3{\cdot}4 \times 10^{-11}$	0·2
Glycollate		300	Soluble	—
Iodate		150	$2{\cdot}2 \times 10^{-7}$	6·0
Iodoacetate		300	Soluble	—
Mandelate		300	Soluble	—
Malate	2	100	$2{\cdot}5 \times 10^{-3}$	50
Malonate		100	$6{\cdot}25 \times 10^{-4}$	25
Oxalate		5	$1{\cdot}8 \times 10^{-9}$	0·06
Succinate		100	$6{\cdot}4 \times 10^{-3}$	80
Sulphate		100	$6{\cdot}1 \times 10^{-5}$	8
Thiosulphate		100	Soluble	1
Tartrate		30	$7{\cdot}7 \times 10^{-7}$	0·9
Citrate	3	5	$1{\cdot}7 \times 10^{-8}$	0·4
Ferricyanide		30	Soluble	—
Methanetrisulphonate		30	Soluble	—
Naphthalenetrisulphonate		20	Soluble	—

tested and the calcium ion concentration of their saturated aqueous solutions. The values of their solubility products are those given in the International Critical Tables (1929) except the value for calcium citrate which was calculated from the data of Schubert & Lindenbaum (1950).

The results show a close connexion between anticoagulant activity and valency for the anions tested. The concentrations of uni-, bi-, and tervalent ions needed to prevent clotting fell in the order 10 : 3 : 1 respectively. There appears to be no direct connexion between anticoagulant activity and ability to remove calcium ions from solution; the concentration of fluoride ions needed to prevent clotting is sixteen times that of citrate ions, although the former are more active in removing calcium from solution. A close examination of the table reveals, however, that all of the anions tested having calcium salts with solubility products less than 10^{-7} were more active in preventing coagulation than were other ions of the same valency.

The concentration of electrolytes and calcium required to cause coagulation of salt-free blood. The results shown in Fig. 1 were obtained by successively adding salts and calcium ions to plasma decalcified and dialysed as described previously. The figure shows that coagulation will not occur if the ionic strength of blood is less than 0·01. As the salt concentration is raised the quantity of calcium required for coagulation falls to a minimum value at an ionic strength of 0·03. When the salt concentration is increased beyond this point there is an approximately proportional increase in the quantity of calcium needed for coagulation until an ionic strength of 0·3 is reached. Beyond this there is an upper limit of ionic strength, varying with salt added, at which coagulation will no longer occur.

For the most part the data from which Fig. 1 was constructed were obtained by adding sodium and calcium chlorides to salt-free plasma. It was found, however, as the diagram shows, that for ionic strengths below 0·10 closely similar results could be obtained by adding sodium sulphate, or potassium ferricyanide in place of sodium chloride. The difference in behaviour of these salts at ionic strengths above 0·10 is to be expected, since their solutions can then no longer be regarded as dilute.

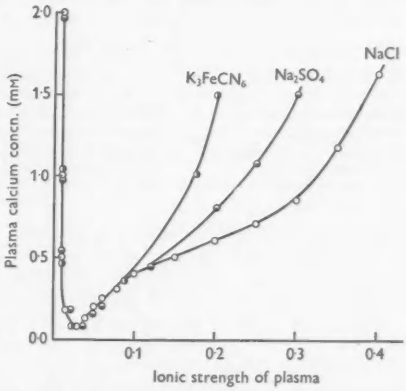


Fig. 1. The calcium concentration required for the coagulation of human plasma of varying ionic strength. Decalcified salt-free human plasma was treated with $CaCl_2$ solution, and a solution of a neutral salt NaCl, Na_2SO_4 or K_3FeCN_6. Tests were made in Pyrex glass tubes at 20° and the calcium concentration needed for coagulation within 12 hr. observed.

The effect of dilution upon the concentration of calcium required for coagulation. The results so far given suggest that the presence of free calcium ions exerts a considerable influence on the coagulation process. They do not, however, provide direct evidence concerning the condition of the element in coagulating plasma, in particular to what extent the element functions in the adsorbed, combined and ionized states. In view of the importance attached by Quick & Stephanini (1948) to the non-ionized calcium in coagulating plasma, the following experiment was made to determine if calcium did function solely in the non-ionized state as Quick & Stephanini suggest.

Table 2. *The concentration of calcium ions required for the coagulation of plasma diluted with varying volumes of salt solution*

(Decalcified, salt-free, human plasma was diluted with 0·15 and 0·03 M-NaCl solution containing Ca ions at the concentrations listed in the table. The coagulation time at 20° in Pyrex tubes was observed.)

Dilution of plasma	Ionic strength of plasma	Coagulation time (hr.) at calcium concentrations (mM)				
		2·0	1·0	0·5	0·2	0·1
1 : 2	0·15	0·5	0·5	4·0	>24	>24
1 : 5	0·15	0·5	0·5	4·0	>24	>24
1 : 10	0·15	0·5	0·5	4·0	>24	>24
1 : 20	0·15	0·5	4·0	4·0	>24	>24
1 : 2	0·03	0·5	0·5	1	4	12
1 : 5	0·03	0·5	0·5	1	4	12
1 : 10	0·03	0·5	0·5	1	4	12
1 : 20	0·03	0·5	0·5	4	4	12

Table 3. *The coagulation of decalcified plasma by various substances*

(Measurements were made of the quantity of test material required to cause the coagulation of decalcified human plasma within 12 hr. at 20° in Pyrex glass tubes.)

Material	Previous treatment	Quantity needed for coagulation in 12 hr. or less (mg./ml.)	Calcium removed by boiling with N-HNO_3 (μg./g.)
Powdered soft glass	Washed with distilled water	20	230·0
Powdered Pyrex glass	Washed with distilled water	200	10·0
Powdered silica glass	Washed with distilled water	200	8·0
Powdered soft glass	Washed with N-HNO_3	No coagulation with 1 g./ml.	—
Powdered Pyrex glass	Washed with N-HNO_3	No coagulation with 1 g./ml.	—
Powdered silica glass	Washed with N-HNO_3	No coagulation with 1 g./ml.	—
Calcium oxalate	Washed with distilled water	0·1	—

Plasma, free of calcium and salts, was prepared as described earlier in this paper. A series of dilutions of this plasma was made in sodium chloride solution containing various amounts of calcium chloride. The coagulation time of the diluted plasma was observed and is recorded in Table 2. The table clearly shows that the coagulation time is dependent upon the concentration of calcium, and not upon the quantity present. This is the reverse of what would be expected if the calcium necessary for coagulation were present solely in the non-ionized condition.

The coagulation of decalcified blood by powdered glass, calcium oxalate and other substances. In experiments described previously (Lovelock & Burch, 1951), it was found that decalcified plasma would often coagulate if kept in soda-glass vessels, or when in contact with powdered glass or silica. Similar observations were reported by Overman (1949), who found that diluted oxalated blood would clot when in contact with powdered glass and other similar materials. At the time it seemed that these observations indicated that coagulation could occur in the absence of calcium. The possibility that sufficient calcium was liberated from the glass, etc., to cause coagulation was not seriously considered, since it was then believed that the minimum concentration of calcium at which coagulation could normally occur was 0·4 mM. It was suggested that the function of calcium and of powdered glass in the coagulation of blood was respectively to combine with, or inactivate, some inhibitor of coagulation. Reference to Fig. 1, however, shows that in blood diluted to an ionic strength of 0·03 the concentration of calcium required for coagulation is only 0·04 mM (1·6 μg./ml.). There seemed a distinct possibility that a quantity of calcium as small as this could be liberated from glassware or powdered glass.

Table 3 shows the quantities of various materials needed to cause the coagulation of decalcified plasma, and the quantity of calcium which could be removed from the same quantity of material by boiling with N-nitric acid for 2 hr. It is evident from the results that sufficient calcium is available from powdered glass and probably also from powdered silica to cause coagulation. That glass and silica washed with acid will not cause coagulation, further confirms that these materials cause the coagulation of decalcified blood by liberating calcium adsorbed upon their surfaces.

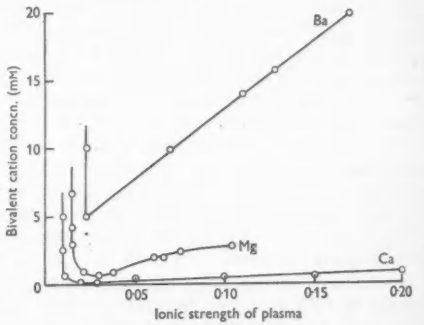


Fig. 2. The concentrations of barium, magnesium and calcium ions required for the coagulation of human plasma of varying ionic strength. Decalcified salt-free human plasma was treated with NaCl solution, and with a solution of $CaCl_2$, $BaCl_2$ or $MgCl_2$. Tests were made in Pyrex glass tubes at 20° and the divalent cation concentration needed for coagulation within 12 hr. observed.

The results shown in Table 3 also include the observation that coagulation can occur when calcium oxalate is added to decalcified blood. This experiment has been repeated successfully on several occasions and with blood from different persons. The calcium oxalate used was precipitated from a solution containing an excess of oxalate ions and washed with dilute potassium oxalate solution before use. It was unlikely, therefore, to have calcium ions adsorbed upon its surface. This result illustrates clearly the fallacy of assuming, as is frequently done, that the precipitated calcium oxalate in oxalated blood is inert.

The coagulation of decalcified plasma by cations other than calcium. Fig. 3 shows the relationship

between the electrolyte content of plasma and the concentration of calcium, magnesium and barium, required for coagulation. The concentration of each of these cations needed for coagulation varied with the ionic strength of the plasma in a closely similar manner. The most favourable ionic strength for coagulation was the same for all three elements, namely 0·03 and above this the concentration of each cation needed for coagulation increased proportionately with increasing ionic strength. The concentrations of calcium, magnesium and barium needed for coagulation rose in the order 1 : 8 : 60 respectively.

The values and sequence of activity of calcium, magnesium and barium ions in the coagulation of plasma are similar to those required to neutralize the charge of certain phosphate colloids. For example, the charge on sphingomyelin is neutralized by calcium, magnesium and barium ions at concentrations of 0·9, 5·0 and 20 mM respectively (Bungenberg de Jong, Teunissen-Van Zijp & Teunissen, 1940). Bungenberg de Jong (1949) has further shown that the activity of bivalent cations in neutralizing the charge on phosphatide colloids is reduced when the ionic strength of the suspending medium is increased.

DISCUSSION

The experimental observations indicate that the coagulation process requires the concentration and proportion of simple electrolytes in plasma to be within a prescribed range. In particular, the process is sensitive to changes in the proportion of the total ionic strength contributed by calcium ions. Coagulation occurs most readily when the proportion is approximately 1 : 16 for calcium and for other ions respectively. The experimental establishment of the idea that a certain electrolyte balance is necessary for normal coagulation has proved of use in resolving some of the discrepancies concerning the function of calcium in coagulation and of oxalate, citrate and fluoride ions in preventing it. For example, the discrepancy between the concentrations of oxalate, citrate and fluoride ions needed for coagulation and their ability to decalcify plasma can be resolved without recourse to complex theories concerning the function of calcium in coagulation. The addition of these ions to plasma disturbs the electrolyte balance in a twofold manner; first by lowering the calcium ion concentration, and secondly by raising the total ionic strength. That citrate ions are more effective than fluoride ions in preventing coagulation then follows as a natural consequence of their higher valency. With oxalate ions their superior decalcifying activity compensates for their lower valency to give them approximately the same anticoagulant activity as citrate ions. That the decalcifying activity of these ions alone is insufficient to prevent coagulation is further illustrated by the observation that decalcified blood can be coagulated by the addition of calcium oxalate, citrate or fluoride. It should be noted that the solubilities of the calcium salts listed in Table 1 refer to their solutions in water only. They are of use in illustrating qualitatively the ability of the anions tested to decalcify blood, but are almost certainly different quantitatively from the solubilities of the same salts in blood, especially in the presence of an anticoagulant concentration of the test anion. Norbdo (1936) has shown, for example, that the calcium ion content of a saturated solution of calcium oxalate in serum ultrafiltrate is 0·18 mM. The calcium ion content of a saturated solution of the same salt in water is 0·06 mM. When precise values of the solubilities of calcium salts in whole blood become available it should be possible, using also the data in Fig. 1, to calculate the concentration of a given anion needed to inhibit the coagulation of blood. The comparison of the calculated and the observed anticoagulant activity of salts such as those listed in Table 1 would provide a critical means of checking the validity of the theories outlined above.

It is frequently stated that the coagulation of citrated or oxalated blood following the addition of calcium is further proof of the necessity of that element for coagulation. In spite of the obvious illogicality of this statement, in that the addition of calcium removes the oxalate and citrate ions, it is of some interest to compare it with the observation illustrated in Fig. 1. Here the addition of calcium ions to plasma, prevented from coagulating by the addition of sodium chloride, also causes coagulation. In both of these examples it is the restoration of the electrolyte balance which restores the coagulability of the plasma.

The experiments show that even with the smallest amount of calcium at which coagulation will occur, namely 0·08 mM, it is the concentration and not the quantity of calcium in plasma which is important. This is not in accord with those theories which postulate that some non-ionized calcium compound is necessary for coagulation. Neither is the evidence that the concentration of calcium required for coagulation varies with the ionic strength of the plasma, nor the observation that the concentrations of other bivalent elements needed for coagulation are different from those of calcium. An acid radical giving a firm non-ionized compound with 1 part of calcium, 8 of magnesium or 60 of barium, seems very improbable. In general terms the experimental evidence does not support the idea that a defined compound of calcium and one of the macromolecules of plasma, presumably analogous to iron in haemoglobin, is necessary for coagulation.

Calcium is distributed in plasma in the form of free ions, ions adsorbed upon the many colloid species present and probably also in non-ionized combination with the simpler anions of plasma. The equilibria controlling the proportion of the element distributed in these various forms are likely to be complex and easily disturbed by changes of the electrolyte and calcium ion content of plasma. In these circumstances the discovery of the precise function, or functions, of calcium in the normal coagulation process is likely to be difficult. Nevertheless, from a knowledge of the nature of the coagulation process, namely the serial interaction of a number of electrically charged macromolecules, the following conclusions can be drawn: calcium ions as such are unlikely to enter directly into the coagulation reaction, but their presence is necessary in order to maintain the requisite amount of calcium adsorbed upon the various plasma colloids. According to the evidence presented earlier, calcium in firm non-ionized combination with either the molecules, or macromolecules of plasma does not appear to be necessary for coagulation.

It follows therefore that the most probable condition of the element calcium necessary for coagulation is the adsorbed state. It seems possible that the function of the adsorbed calcium is to maintain the surface charge of one or more of the plasma colloids at a value suitable for its proper interaction with the other plasma colloids. There is as yet no direct evidence to suggest which of the plasma colloids require adsorbed calcium for their proper function, although the sequence and degree of activity of calcium, magnesium and barium ions suggests that a phosphate colloid may be involved. Overman (1949) has suggested from other evidence that calcium may function by neutralizing a phosphatide inhibitor of coagulation.

SUMMARY

1. Experiments are described which indicate that the activity of oxalate, citrate and fluoride ions in preventing coagulation is complex, and is due not only to their ability to depress the calcium ion concentration of blood, but also to their ionic charge.

2. The concentrations of calcium, magnesium and barium ions needed for the coagulation of plasma containing various concentrations of salts has been measured. These measurements indicate that human plasma will not coagulate if the ionic strength is less than 0·01 or more than 0·5. The concentration of alkaline earth elements required for coagulation was found to vary with ionic strength, calcium concentration as low as 0·08 mM causing coagulation in plasma of ionic strength 0·03.

3. The function of calcium in coagulation is discussed. It is suggested that it is concerned with the maintenance of an electrolyte balance suitable for the interaction of the plasma colloids.

REFERENCES

Arthus, M. & Pagés, C. (1890). *Arch. Physiol. norm. path.* **2**, 739.

Astrup, T. (1944). *Acta physiol. Scand.* **7**, 11.

Bungenberg de Jong, H. G. (1949). *Colloid Science.* London: Elsevier.

Bungenberg de Jong, H. G., Teunissen-Van Zijp, L. & Teunissen, P. H. (1940). *Kolloidzschr.* **91**, 311.

Fuld, E. & Spiro, K. (1904). *Beitr. chem. Physiol. Path.* **5**, 171.

Glazko, A. J. & Greenburg, D. M. (1939). *Amer. J. Physiol.* **125**, 108.

International Critical Tables (1929). **4**, 229–31.

Jacques, L. B., Fidlar, E., Feldstedt, E. T. & Macdonald, A. G. (1946). *Can. med. Ass. J.* **55**, 26.

Lovelock, J. E. & Burch, B. M. (1951). *Biochem. J.* **48**, xxxiv.

Mommaerts, W. F. H. M. (1945). *J. gen. Physiol.* **29**, 103.

Morawitz, P. (1904). *Beitr. chem. Physiol. Path.* **5**, 133.

Norbdo, R. (1936). *Skand. Arch. Physiol.* **75**, Suppl. 11.

Overman, R. S. (1949). *Blood Clotting and Allied Problems.* New York: J. Macey.

Pekelharing, C. A. (1892). *Dtsch. med. Wschr.* **18**, 1133.

Quick, A. J. & Stephanini, M. (1948). *J. gen. Physiol.* **32**, 191.

Sabbatani, L. (1908). *Mem. R. Accad. Torino*, **51**, 257.

Schmidt, A. (1895). *Weitere Beitrage zur Blutlehre.* Wiesbaden: Bergmann.

Schubert, J. & Lindenbaum, A. (1950). *Nature, Lond.*, **166**, 913.

Steinberg, A. (1944). *Proc. Soc. exp. Biol., N.Y.*, **56**, 124.

Stephanini, M. (1948). *Proc. Soc. exp. Biol., N.Y.*, **67**, 22.

Stewart, C. P. & Percival, G. (1928). *Biochem. J.* **22**, 559.

Vines, H. W. C. (1921). *J. Physiol.* **55**, 86.

Enzyme Inhibition by Suramin and the Measurement of the Isoelectric Points of some Enzymes

By E. D. WILLS
Department of Biochemistry and Chemistry, Medical College of St Bartholomew's Hospital, London

(*Received* 12 *April* 1951)

During a study of the action of the trypanocidal drug, suramin, on various enzymes it was observed that the inhibitory effect of the drug on some enzymes was dependent on the pH of the solution. Thus, whereas M/3000 suramin almost completely inhibited urease at pH 5·0, no inhibition occurred at pH 5·3, even when much higher concentrations were used (Wills & Wormall, 1949). In further investigations it was found that with several other enzymes there was a similar sharp drop in the inhibition-pH curve at or near the isoelectric point of the enzyme concerned. As a result of these observations it was suggested that, where an enzyme is inhibited by suramin and where the inhibition shows the above-mentioned marked dependence on the pH of the medium, 'suramin-inhibition' tests might give valuable information about the isoelectric point of the enzyme (Wills & Wormall, 1950*a*).

A study of a wide range of suramin-sensitive enzymes has fully confirmed this view, for there is very good agreement between the values obtained by the suramin-inhibition method and corresponding values recorded in the literature for the isoelectric points of most of these enzymes. Brief preliminary reports of some of these results have been given elsewhere (Wills & Wormall, 1950*b*, *c*). Further results, and full details of the method, are given in this paper.

EXPERIMENTAL

Enzyme preparations. The methods of preparing the various enzymes and the measurement of their activity have been described previously (Wills & Wormall, 1950*a*). The sources of some enzymes are recorded in Table 1. The sources of others were as follows: urease (soya bean and jackbean), peroxidase (horse-radish), ascorbic acid oxidase (cucumber and cabbage), invertase (a commercial preparation from yeast), β-glucosidase (ground almond meal).

Buffer solutions. Acetate buffer was used for the pH range 3·8–5·7, and phosphate buffer for the pH range 5·7–6·5. Both series were prepared to the formulae of Clark and Lubs. The pH of all buffer solutions was measured with the glass electrode before use and the pH of each mixture was checked with indicators at the end of the experiment.

Method

The method consisted of estimating the enzymic activity with and without suramin over a range of pH. The percentage inhibition was then plotted against pH. Where the method is of value, the curve falls sharply, from about 100 to 10% or less, over a range of 0·3–0·8 of a pH unit, and the midpoint of this range, or probably more accurately that of the range covering 10–90% inhibition, is regarded as the mean isoelectric point of the enzyme. It was usually possible to measure this value to within 0·2, and sometimes to the nearest 0·1 of a pH unit. With some enzymes, the drop in the pH-inhibition curve covered a range of a whole unit of pH or more and it is suggested that in these cases less precision should be given to the '50% inhibition' value. Sometimes, however, this slow fall of the curve can be attributed to the presence of two different but similarly acting enzymes (e.g. α- and β-amylases) in the preparation studied.

M/1000 suramin was used in almost every case, since this concentration was found to give the sharply dropping type of curve required. More dilute solutions gave less steep curves, whilst higher concentrations of suramin (e.g. M/500), although not exhaustively investigated, appeared to offer no advantage over M/1000. The suramin should be left in contact with the enzyme for a standard period of time before the addition of the substrate; a period of 20 min. is satisfactory (Wills & Wormall, 1950*a*).

Since these factors are known to affect the values obtained for the isoelectric point of a protein by conventional methods the type of buffer used and its concentration have been specified in each case. An exhaustive study of the effect of changing the type of buffer and salt concentration has not yet been made, but preliminary experiments have shown that these changes have no marked effect on the results obtained by the suramin-inhibition method, possibly on account of the relative insensitivity of the method.

RESULTS

Effect of pH *on the inhibition of various enzymes by suramin*

In view of the results obtained with urease (Wills & Wormall, 1949, 1950*a*), similar tests were made with several other enzymes and many were found to give curves of the same type as that given by urease. With many enzymes it has been possible to confirm that the curve drops sharply at the known isoelectric point of the enzyme. In other cases, however, no authentic value for the isoelectric point, as determined by electrophoresis, appears to be available in the literature and the values obtained by the suramin-inhibition method with those enzymes should be regarded as tentative; they are presented here so that they can subsequently be compared with the results which may be obtained by electrophoresis of the crystalline enzymes.

Table 1. *Isoelectric points of some enzymes measured by the 'suramin-inhibition' method and compared with values recorded in the literature*

Enzyme	Buffer* (and concn.)	Isoelectric point: Suramin method (pH)	Recorded values: For crystalline enzyme (pH)	Recorded values: For non-crystalline enzyme (pH)	Reference
Urease	Acetate	5·1	5·0–5·1	—	Sumner & Hand (1929)
Catalase:					
Ox liver	Acetate or phosphate	5·8	5·7	—	Sumner & Dounce (1937)
Sheep blood	Phosphate	6·2	—	—	—
Rabbit blood	Acetate	5·2	—	—	—
Human blood	Acetate	5·0	—	—	—
Horse liver	Acetate	5·2–5·3	5·4	—	Agner (1942)
Horse blood	Acetate	5·2–5·3	—	—	—
Amylase:					
Human saliva (α)	Acetate (0·03 M)	5·3	5·0–5·5‡	—	
Malt (α)	Acetate (0·02 M)	5·0	5·7	—	Fischer & Haselbach (1951)
			—	5·75	Daniellson & Sandegren (1947)
Malt (β)	Acetate (0·02 M) or phosphate (0·012 M)	5·7	—	5·75	Daniellson & Sandegren (1947)
Hog pancreas (α)	Acetate (0·05 M)	5·2	5·2–5·6	—	Fischer & Bernfeld (1948)
Carbonic anhydrase:					
Sheep blood	Acetate or phosphate	5·8–6·0	—	—	—
Ox blood	Acetate	5·2–5·4	—	5·3	Petermann & Hakala (1942)
Peroxidase	Acetate	4·9	7·2	—	Theorell (1943)
Tyrosinase:					
Potato	Acetate	4·3	—	5·4	Kubowitz (1937)
Mushroom	Acetate	3·9†	—	Below 5·0	Nelson & Dawson (1944)
Ascorbic acid oxidase	Acetate or phosphate	5·1	—	—	—
Invertase	Acetate (0·05 M)	4·1	—	4·42	Dieu (1947)
β-Glucosidase	Acetate	4·4	—	5·7–5·8	Edman & Jorpes (1941)

* 0·067 M-Acetate or 0·016 M-phosphate, unless some other concentration is indicated.
† Using catechol as substrate.
‡ 'Very probable value' (Meyer, 1949).

Table 1 gives a list of enzymes so far investigated by the suramin-inhibition method and a comparison of the results with those obtained by standard methods for the determination of isoelectric points.

Typical inhibition-pH curves are given in Fig. 1. The '50%-inhibition' point (pH 5·2) for pig pancreatic α-amylase agrees well with the recorded isoelectric point. Human salivary amylase and malt α- and β-amylases gave curves of the same type, and in two cases the pH of the midpoint of the inhibition-pH curve was almost identical with the recorded isoelectric point; in the case of malt α-amylase, however, the suramin inhibition method gave a value of pH 5·0, whereas Fischer & Haselbach (1951) give pH 5·70 as the isoelectric point of this enzyme. It has not so far been possible to explain the difference between the two values.

Invertase also gives a sharply dropping curve, with midpoint about pH 4·1 (Fig. 1), but in this case no comparison with an established authentic isoelectric point is possible, since the crystalline enzyme does not appear to have been prepared. However, Dieu (1947) as a result of careful electrophoretic studies on purified invertase has concluded that this preparation consisted of two closely associating components. Only one of these was found to possess invertase activity and the isoelectric point of this component was determined as being 4·42, which compares reasonably well with the value found by the suramin method (pH 4·1). Quastel & Yates (1936) obtained a curve similar to that in Fig. 1 in their study of the action, on invertase, of several acidic dyes related to suramin, but they did not extend the investigation to other enzymes.

The inhibition of invertase by suramin in acid solution differs from that of urease in that it can be reversed by adding alkali to change the pH of the mixture from 3·8 to 5·1.

Peroxidase gave a similar sigmoid-type inhibitor-pH curve, but the isoelectric point indicated by the suramin method (pH 4·9) differs appreciably from the value (pH 7·2) quoted by Theorell (1943). It is possible in this case that suramin combines with the haematin group of the enzyme. If inactivation

occurs by such a mechanism it would invalidate any figure obtained for the isoelectric point of the enzyme using the suramin method.

Edman & Jorpes (1941) have partially purified emulsin and state that they obtained an electrophoretically homogeneous fraction which possessed emulsin activity and had an isoelectric point of pH 5·7–5·8; the preparation was not pure β-glucosidase, however, and it showed high phosphatase activity. The value of 5·7–5·8 differs considerably from that found by the suramin method (pH 4·4–4·5), but it is possible that better agreement would be obtained if an electrophoretic study were made on the crystalline enzyme.

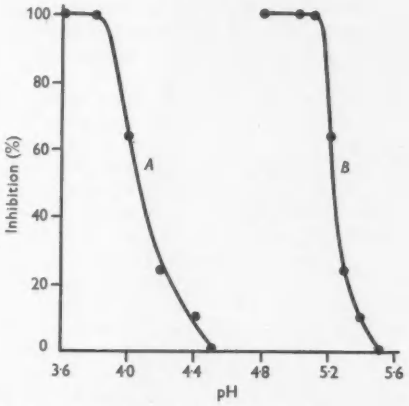


Fig. 1. Effect of pH on the inhibition of α-amylase (hog pancreas) and invertase by suramin. 0·001 M-Suramin; drug and enzyme left in contact for 20 min. before the addition of substrate. *A*, invertase; *B*, α-amylase.

Ascorbic acid oxidase yields a rather unusual type of curve which drops sharply at about pH 5·1, but remains fairly constant at about 40 % inhibition as the mixture is made more alkaline (i.e. over the range pH 5·5–8·0). It is, of course, possible that the enzyme preparation used contained two enzymes capable of oxidizing ascorbic acid, and that only one of them is inhibited by suramin.

For the experiments with tyrosinase (monophenol and polyphenol oxidases) two substrates, *p*-cresol and catechol, were used. Potato tyrosinase gave similar but not quite identical inhibition-pH curves with the two substrates, but mushroom tyrosinase gave two different curves (*A* and *B*, Fig. 2). It is tentatively suggested that these results indicate that the oxidation of catechol by mushroom extracts is catalysed by one enzyme, whereas that of *p*-cresol may be due to two or more enzymes, only one of which is a true phenoloxidase (cf. Keilin & Mann, 1938).

Catalases gave inhibition-pH curves similar to that obtained with urease. Of the six catalases examined, however, those of horse blood and horse liver gave curves which were almost identical, with a midpoint of pH 5·2–5·3; the other four (those of human, rabbit and sheep blood and ox liver) gave curves which differed amongst themselves, and also from that for horse blood and liver. Recorded values for the isoelectric points of catalases are pH 5·7 for the crystalline ox-liver enzyme (Sumner & Dounce, 1937) and pH 5·4 for the crystalline horse-liver enzyme (Agner, 1942). The suramin-inhibition method gave pH values of 5·8 and 5·2–5·3 respectively, for these two enzymes. Bonnichsen (1947) reported that horse-liver and horse-blood catalases are identical, and it is interesting to note that these two enzymes give essentially the same inhibition-pH curve with suramin.

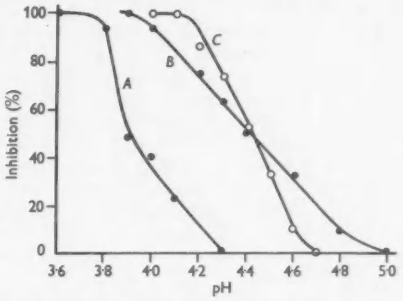


Fig. 2. Effect of pH on the inhibition of tyrosinase by suramin. Mushroom tyrosinase acting on catechol (*A*) and *p*-cresol (*B*). Potato tyrosinase acting on either catechol or *p*-cresol (*C*).

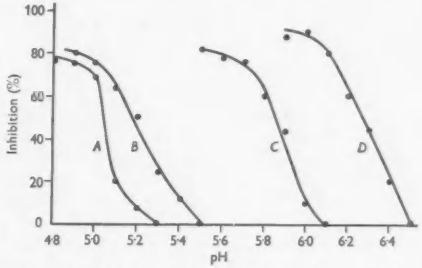


Fig. 3. Effect of pH on the inhibition of various catalases by suramin. Catalases of human blood (*A*), rabbit blood (*B*), ox liver (*C*) and sheep blood (*D*).

Evidence of species differences between the carbonic anhydrases, with respect to their inhibition by suramin, was obtained in experiments with the ox- and sheep-blood enzymes. The inhibition-pH curves for these two enzymes do not drop as sharply

as do those of urease, the catalases, etc., mainly because of the difficulty in estimating the activity of the enzyme in acid solution.

Inhibition of crude enzymes by suramin

Only in the case of urease has it been possible to compare the inhibition-pH curves of the crystalline and impure enzymes. Almost identical curves were obtained with crystalline jack-bean urease and the crude enzyme (soya-bean extracts); considerably more suramin is, however, required to give complete inhibition with crude enzyme preparations, owing to the protective action of the protein impurities. Furthermore, it has been found that the addition of unrelated proteins, such as ovalbumin or those of rabbit serum, has no significant effect on the inhibition-pH curve for urease, though here also more suramin has to be used in the tests.

These results led us to suggest that the method might have a special value in that it is applicable to crude enzyme preparations (Wills & Wormall 1950*a*, *b*). Indeed it may sometimes be possible to obtain an indication of the presence of two similarly acting enzymes in an enzyme preparation. With malt extract the amylase inhibition-pH curve slopes gradually over the range pH 4·6–6·3, whereas the separated α- and β-amylases both give sharply falling curves covering the ranges pH 4·6–5·3 and 5·4–6·3 respectively. The curve for the crude malt extract thus appears to be a resultant of the curves for α- and β-amylases.

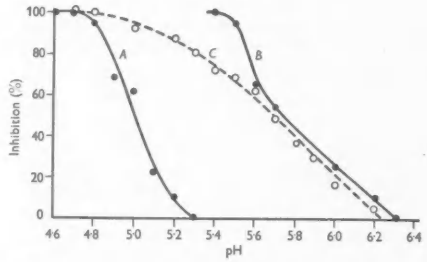


Fig. 4. Effect of pH on the inhibition, by suramin, of the separated malt α- and β-amylases and the amylases in a crude malt extract. α-Amylase (*A*), malt extract (*C*) and β-amylase (*B*).

DISCUSSION

This suramin inhibition method for the estimation of enzyme isoelectric points, although it has a limited application, may be of value in many enzyme studies. The method has, in fact, already been used by Smith & Mills (1950) to determine the isoelectric point of the β-glucuronidase of *Escherichia coli*. It has not the accuracy, reliability or the general applicability of the classical physical methods such as electrophoresis, but it has several interesting and important features. (1) It is easily carried out with simple equipment, usually in a period of a few hours. (2) Whereas the standard methods require extremely pure and preferably crystalline enzymes, the suramin inhibition method can be satisfactorily used, where the method is applicable, with crude enzyme preparations. (3) Values obtained with impure enzyme preparations may serve as a guide for the purification of an enzyme by precipitation at various pH values. (4) The slope of the curve may sometimes indicate that two or more similarly acting enzymes are present in the preparation, as, for example, with the amylases and the phenoloxidases. (5) It may sometimes give a value for the isoelectric point of an enzyme which, in a pure state, is unstable at its isoelectric point (e.g. tyrosinase).

It must be emphasized, however, that the suramin inhibition method is not applicable to the following enzymes. (1) Those which are inhibited by suramin independently of the pH of the medium ('group A' enzymes; Wills & Wormall, 1950*a*). (2) Proteolytic enzymes, when protein substrates are used. The drug readily combines with most, or even all, proteins (cf. Boursnell & Wormall, 1939; Town, Wills, Wilson & Wormall, 1950) and this undoubtedly influences the tests for protease inhibition. The use of short-chain peptides and their derivatives might overcome this difficulty, and this possibility is being explored (Town & Wormall, unpublished). (3) Any enzyme whose activity falls sharply on the acid and alkaline sides of its optimum pH.

The possibility that suramin inhibits many enzymes by a 'bridging' effect has been previously discussed (Wills & Wormall, 1950*a*). If this hypothesis is correct and suramin combines with basic groups in the neighbourhood of the active centre of the enzyme, it will be clear that the method described here can only give a measure of the isoelectric point of that part of the molecule near the active centre, whilst standard physical methods give an isoelectric point for the molecule as a whole. Since there is good agreement between the two methods in several cases, this suggests that there is no abnormal arrangement of acidic or basic groupings in the neighbourhood of the active centres of these enzymes.

The literature dealing with enzymes, even that of recent years, usually tends to give an impression that enzymes which carry out the same function, providing they can be isolated completely pure, are chemically identical, irrespective of the source from which they are obtained. That this view is not entirely correct has been demonstrated by the work of Bonnichsen (1947) on catalases and by Meyer

and his colleagues (cf. Meyer, Fischer, Bernfeld & Duckert, 1948) on the amylases, for these investigators have obtained clear evidence of species differences. Additional support for their observations has been provided by the present experiments, where it has been possible to demonstrate species differences with catalases, amylases and carbonic anhydrases (cf. also Wills & Wormall, 1950*b*, *c*). For example, when tested under identical conditions, four catalases gave four distinctly different curves, with '50% inhibition' or isoelectric points of pH values of 5·0, 5·2, 5·8 and 6·2.

SUMMARY

1. Further details are given of the use of inhibition tests with suramin for the measurement of the isoelectric points of certain enzymes (Wills & Wormall, 1950*a*, *b*). The method is simple and rapid, and can be carried out with impure enzyme preparations.

2. This suramin-inhibition method has been used to measure the isoelectric points of nineteen enzymes. Where comparison is possible (fourteen enzymes) there is, in general, good agreement between the values obtained and those recorded in the literature for electrophoretic examination of the pure enzymes.

3. The results support the view that there are marked species differences amongst the catalases, amylases and carbonic anhydrases.

I am indebted to Prof. A. Wormall for his advice and interest in this work. Some of the expenses of these investigations were covered by a grant to Prof. Wormall from the Medical Research Council.

REFERENCES

Agner, K. (1942). *Ark. Kemi Min. Geol.* **16A**, no. 6.
Bonnichsen, R. K. (1947). *Arch. Biochem.* **12**, 83.
Boursnell, J. C. & Wormall, A. (1939). *Biochem. J.* **33**, 1191.
Daniellson, K. E. & Sandegren, E. (1947). *Acta chem. Scand.* **1**, 917.
Dieu, H. (1947). *Bull. soc. chim. Belg.* **55**, 327.
Edman, P. & Jorpes, E. (1941). *Acta physiol. scand.* **2**, 41.
Fischer, E. H. & Bernfeld, P. (1948). *Helv. chim. Acta*, **31**, 1831.
Fischer, E. H. & Haselbach, C. H. (1951). *Helv. chim. Acta*, **34**, 325.
Keilin, D. & Mann, T. (1938). *Proc. Roy. Soc.* B, **125**, 187.
Kubowitz, F. (1937). *Biochem. Z.* **292**, 221.
Meyer, K. H. (1949). Private communication.
Meyer, K. H., Fischer, E. H., Bernfeld, P. & Duckert, F. (1948). *Arch. Biochem.* **18**, 203.
Nelson, J. M. & Dawson, C. R. (1944). *Advanc. Enzymol.* **4**, 99.
Petermann, M. L. & Hakala, N. V. (1942). *J. biol. Chem.* **145**, 701.
Quastel, J. H. & Yates, E. D. (1936). *Enzymologia*, **1**, 60.
Smith, E. E. B. & Mills, G. T. (1950). *Biochem. J.* **47**, xlix.
Sumner, J. B. & Dounce, A. L. (1937). *J. biol. Chem.* **121**, 417.
Sumner, J. B. & Hand, D. B. (1929). *J. Amer. chem. Soc.* **51**, 1255.
Theorell, H. (1943). *Ergebn. Enzymforsch.* **9**, 231.
Town, B. W., Wills, E. D., Wilson, E. J. & Wormall, A. (1950). *Biochem. J.* **47**, 149.
Wills, E. D. & Wormall, A. (1949). *Biochem. J.* **44**, xxxix.
Wills, E. D. & Wormall, A. (1950*a*). *Biochem. J.* **47**, 158.
Wills, E. D. & Wormall, A. (1950*b*). *Nature, Lond.*, **165**, 813.
Wills, E. D. & Wormall, A. (1950*c*). *J. Physiol.* **111**, 13*P*.

Astaxanthin, Cholesterol and Lipins in Developing Salmon Eggs

By MARY GLOVER, R. A. MORTON and D. G. ROSEN
Biochemistry Department, The University of Liverpool

(*Received* 25 *April* 1951)

In this paper observations on provitamins and vitamins A and D and the carotenoid astaxanthin in developing salmon eggs are recorded, together with changes in neutral fat, cholesterol and phospholipin content. An adequate discussion of the broader aspects of these matters must await more facts pertaining to more species.

This study shows among other things a marked conservation of astaxanthin during development and a striking lack of evidence that vitamin A (or A_2) is required in more than traces, if at all.

Through the kindness of Dr R. J. Daniel (until recently of the Department of Oceanography in this University), supplies of salmon eggs were made available to us over a total period of some 3 months in one season and at intervals in previous years.

EXPERIMENTAL

Materials

Salmon eggs (Salmo salar). The eggs were kept in trays in a shallow tank through which water at 9–10° was kept flowing. The eggs were fertilized early in December; about

70% survived until near the end of the yolk sac period. The greatest mortality occurred during 2 days just before hatching. All material was provided by Dr Daniel.

Stages of development. Daniel (1947) has discussed the development of salmon eggs and has used a numerical description correlated with the number of days before ($-x$) and after ($+x$) hatching. His designation of stages of development has been followed here (see Table 1).

Examination of eggs and larvae

Methods of extraction. The larvae (both before and after hatching) were superficially dried with blotting paper and weighed. At different developmental stages eyes were dissected out whole and extracted after thorough grinding. When the embryos and yolks were separated by dissection they were bulked and the average weights recorded (Table 1). In all cases the material was thoroughly ground with five times its weight of anhydrous Na_2SO_4 and the mixture was left to stand under freshly redistilled ether for 2·5 hr. The yellow ether extract was decanted through a G4 sintered-glass filter and the residue washed with warm ether two or three times. The combined extract and washings were taken down to dryness under reduced pressure at room temperature and in a dim light. The lipid residue was weighed and then dissolved in a suitable volume (10–50 ml.) of light petroleum for estimation of the carotenoids (lipid extract I).

The tissue residue, after the first extraction, was then left to stand for 3 hr. under a mixture of equal volumes of ethanol and ether. The supernatant liquid was decanted off and the residue washed as described above. The combined washings and extract were reduced in volume, transferred to a small flask and taken to dryness on a hot-water bath. A few ml. absolute ethanol were added and a stream of N_2 passed through the flask to drive off the last traces of solvent. This second lipid extract II was retained.

Analytical methods

Carotenoids and vitamin A. The light petroleum extract (I) above was examined spectrophotometrically by the method previously reported (Morton & Rosen, 1949).

Phospholipins. In many experiments the greater part of both extracts was used for determining acetone-insoluble lipins. A suitable fraction of the light petroleum extract I was placed in a tared 50 ml. conical flask and taken to dryness. Acetone (35 ml.) was added and the mixture heated to boiling; a small oily residue at the bottom of the flask was well dispersed by shaking. The flask was then allowed to cool and left in the ice chest overnight. The insoluble phospholipins adhered to the wall of the flask so that the supernatant liquid could be decanted off cleanly and the residue washed with a little cold acetone. The insoluble residue was dried on the water bath by adding a little absolute ethanol which was later removed in a stream of N_2. Traces of residual solvent were always removed under suction prior to weighing. The whole of the lipid extract II above was treated with acetone as described, yielding another fraction (2) of phospholipin, enabling the total phospholipin in the tissue to be assayed. The egg capsules may not on every occasion have been quite completely extracted as they were very resistant to grinding.

Cholesterol. This was determined gravimetrically as the digitonide by standard methods either on acetone-soluble extracts or on unsaponifiable fractions. The Liebermann-Burchard reaction was used as a guide to the presence of steroid.

RESULTS

Lipins

Table 1 shows that initially the eggs contained 6·2% of acetone-soluble lipid, and 2·9% of phospholipin. The eggs at spawning contain about 50% of water (Hayes & Hollett, 1940) so that (corrected for water) the results agree well with those of Halpern (1945) who found 12·5% fat and 6·2% phospholipin in salmon roe. Hayes & Ross (1937) at stage I recorded 9·1 mg. true fat (i.e. 7%) for a 'standard hypothetical egg' weighing 130 mg.

Table 1 shows that a gradual, small loss of fat occurs before hatching; this fits the results of respiration studies by Hayes & Hollett (1946), but does not confirm their finding of a small amount of fat synthesis before hatching. At stage XII (45 days

Table 1. *Changes in lipid, astaxanthin and steroid content during development of salmon eggs*

Stage	No. and egg fraction used	Average wt. (g.)	Lipid/individual egg: Acetone-soluble (mg.)	Lipid/individual egg: Acetone-insoluble (mg.)	Astaxanthin (μg./individual egg)*	Steroid (mg./individual egg)
I (−42)†	98 (whole eggs)	0·155	9·61	4·55	0·83	0·45
III (−34)	60 (whole eggs)	0·167	9·32	4·01	0·88	0·81
V (−7)	92 ‡(whole eggs)	0·143	9·01	4·32	0·81	0·60
	(yolks)	0·125	8·48	3·58	0·74	—
	(embryos)	0·018	0·53	0·74	0·08	—
VII–VIII (+14)	73 (whole larvae)	0·116§	8·55	2·67	0·77	2·25
	(yolks)	0·074	7·87	2·25	0·60	—
	(embryos)	0·042	0·68	0·42	0·16	—
XII (+45)	98 (whole larvae)	0·156	5·47	3·47	0·76	0·55
	(yolks)	0·023	3·01	2·13	0·15	—
	(embryos)	0·133	2·46	1·34	0·60	—

* The amounts given are approximate in an absolute sense but are quite comparable relatively.
† Numbers in brackets are days before (−) and days after (+) hatching.
‡ By addition of results on yolks and embryos.
§ Eyes were removed at this stage and some loss of water during the time occupied in dissection may have contributed to the apparent loss of weight.

after hatching) more than 40 % of the fat has disappeared, but there is no concomitant increase in phospholipin content. It is not known whether the fat is utilized, directly or indirectly, for the formation of carbohydrate. The fat content of the salmon embryos shows a rise soon after hatching, although there is an overall loss of fat in the larvae. The phospholipin decrease in both embryo and yolk (+14 days) is difficult to explain on any basis other than utilization for cholesterol synthesis (see p. 428).

Carotenoids

Chromatographic tests showed that young salmon eggs contained neither carotenes nor xanthophylls (it was unfortunately not possible to spare material for examining mature unspawned ova) and astaxanthin, the only carotenoid to be detected, was present almost entirely in the free (unesterified) state. The loss of astaxanthin during 87 days (−42 to +45) was 12%; 71 % was transferred to the embryo and 17 % remained in the yolk. The corresponding figures for fat were: lost 42%, stored in embryo 26%, left in yolk sac 32%. It seems, therefore, that the transport and metabolism of astaxanthin and fat must be largely independent. The astaxanthin in the yolk remained free throughout the period, but in the final determination practically all the astaxanthin of the embryo was epiphasic, i.e. esterified; the embryos also contained an unknown ethanol-soluble substance with λ_{max} 418 mμ. and an inflexion near 500 mμ.

Lipochromes of the eye

The most significant observation was that neither vitamin A_1 nor vitamin A_2 could be detected by the antimony trichloride colour test or by ultraviolet absorption. Fourteen days after hatching the eyes contained about 7 % of the total astaxanthin. In one batch, 200 larvae approaching the end of the yolk sac stage were dissected and the bulked eyes tested for vitamins A by the colour test. A very faint colour with $\lambda_{max.}$ 640 mμ. was recorded, but corresponded with neither the 620 mμ. peak for vitamin A_1 nor the 693 mμ. peak for vitamin A_2. The latter band is subject to colour test inhibition and a band with $\lambda_{max.}$ 640–650 mμ. is sometimes seen. Even if this means the presence of a mere trace of vitamin A_2, the minuteness of the amount is striking.

Cholesterol

The findings for cholesterol (total and free) are shown in Table 2. At no stage was an appreciable amount of 7-dehydrocholesterol found, although a trace may possibly have been present in the acetone-soluble portion of the ethanol-ether extract at stage I (−42 days). The selective absorption at 254 and 261 mμ. at Stage XII (+45 days) has not yet been accounted for (Table 3).

Table 2. *Changes of steroid (precipitable as digitonide) content of developing salmon eggs*

Stage and egg fraction used	Total steroid		Free steroid	
	(mg./individual egg)	(mg./g. tissue)	(mg./individual egg)	(mg./g. tissue)
I (−42)	0·54	3·5	0·51	3·3
III (−34)	0·81	4·8	—	—
V (−7) (yolk sac)	0·55	4·4	0·55	4·4
V (−7) (embryo)	0·05	2·8	0·05	2·8
VIII (+14) (yolk sac)	1·60	21·6	1·26	17·0
VIII (+14) (embryo)	0·65	15·5	0·05*	1·2
XII (+45) (yolk sac)	0·19	8·3	0·13	5·7
XII (+45) (embryo)	0·36	2·7	0·25	1·9

* Difference between total and ester; direct determination gave a negligible free cholesterol value.

Table 3. *Ultraviolet absorption spectra of extracts from developing salmon eggs*

(~ Denotes marked inflexion.)

Stage and egg fraction used	Ether extract	Acetone-soluble portion of ethanol-ether extract
I (−42)	~265–285 mμ.	Max. 270 mμ., weak bands 280 mμ., 290 mμ.
III (−34)	~265–285 mμ. (also shown by 'unsaponifiable' fraction)	Continuous end absorption
V (−7) (yolk)	~265–275 mμ.	~265–280 mμ., Dark-green colour in Liebermann-Burchard test
V (−7) (embryo)	End absorption	End absorption, very-pale colour
VIII (+14) (yolk)	~265–285 mμ.	—
VIII (+14) (embryo)	Feeble inflexion	—
XII (+45) (yolk)	~260–285 mμ.	Material all used in cholesterol determinations
XII (+45) (embryo)	Max. 254, 261 mμ.	—

DISCUSSION

Miescher (1866) recorded that the salmon embryonic system loses 60 % of its egg fat between fertilization and disappearance of the yolk sac. More recent work (Hayes, 1930; Hayes & Ross, 1937) indicates that utilization of fat as fuel occurs mainly in the last 4 weeks. The total fat of the embryo at first increases rapidly, decreases at hatching and then increases steadily. The amount of fat in the yolk sac rises before hatching and then falls slowly until increasing combustion sharply accelerates the rate of disappearance.

Lovern (1934, 1936) found that the fat from ripe salmon eggs contained a larger proportion of unsaturated fatty acids than did salmon depot fat. Anno (1940) observed that the unsaponifiable matter of salmon eggs (*Oncorhynchus gorbuscha*) was nearly all cholesterol. Steven (1947) obtained astacene from the ovaries of the brown trout (*Salmo trutta*). Salmon egg oil shows selective absorption in the region 260–270 mμ., but vitamin A is not present (Johnstone & Hayes, 1938–42). Studies on carbohydrate (Hayes & Hollett, 1940) protein (Hayes & Hollett, 1946) and glycogen (Daniel, 1947) in salmon eggs have also been made.

The present work shows that in the developing salmon egg the principal carotenoid pigment is astaxanthin which is smoothly but only in part transferred to the embryo and esterified. There is not much loss. The absence of detectable amounts of provitamins or vitamins A is striking, especially from the eyes, and points to the need for a special study of the biochemistry of vision in the embryos and of the biological role of astaxanthin.

Both neutral fat and phospholipin appear to be metabolized to a substantial extent during development, since there is an overall loss of both. Transfer to the embryo appears to take place on a considerable scale in the later stages. After hatching (-7 to $+14$ days) there is a sharp increase in both free and combined cholesterol. That this is real is shown by the good agreement between determinations made before and after saponification (Table 2). The rise (about 1·65 mg./individual) corresponds with a small decrease in neutral fat (about 0·45 mg./individual) and a larger decrease in phospholipin (about 1·65 mg./individual) making it appear that the cholesterol is synthesized largely, if not entirely, from phospholipin.

The rise in cholesterol content at stage VIII ($+14$ days) is just as notable for the yolk sac as the embryo, but in the former the cholesterol is mainly free, whereas in the latter a larger proportion is esterified. It seems almost certain that the cholesterol is synthesized in the yolk sac-cell system and then some of it becomes esterified on absorption and storage in the liver of the embryo. In the course of phospholipin breakdown (shown to occur in the case of the hen egg by Hevesy, Levi & Rebbe, 1938) fatty acids and metabolites, therefore, must be used for the synthesis. From stage VIII ($+14$ days) onwards the cholesterol content of the embryo is barely maintained.

The general picture for other species is on the whole consistent with the findings: Hanes (1912), studying hen eggs by histochemical methods, observed that at the fourteenth day of incubation, cholesterol esters began to accumulate in the chick embryo probably at the expense of the phospholipid of the yolk. There is marked absorption of phospholipid from the yolk and yolk sac during the final week of incubation (Riddle, 1916). According to Mueller (1915), the total cholesterol does not change during incubation, but up to the thirteenth day free cholesterol is predominant and thereafter up to 40 % becomes esterified. Roffo & Azaretti (1926) doubted whether cholesterol synthesis takes place, but Channon (1925), Thannhauser & Schaber (1923) and Dam (1928) all considered that there was definite evidence of synthesis; in terms of dry weight of embryo the cholesterol content is at its highest on the eleventh day. Rittenberg & Schoenheimer (1937), however, using deuterium, found neither synthesis nor degradation of cholesterol during incubation of hen eggs up to the twentieth day, but accepted active metabolism of cholesterol as occurring immediately after hatching. Yamaguchi (1924) found storage of cholesterol esters in the embryonic liver of man, rabbit, dog, guinea pig and toad, but curiously not in snakes and salmon; the present work removes the discrepancy with salmon. The occurrence of some cholesterol esters in the yolk sac at stage VIII ($+14$ days) is consistent with the results of Hanes on chick eggs and with the view of Needham (1931) that some esterification is to be expected.

SUMMARY

1. Over a period of about 3 months from fertilization developing salmon eggs, examined at different stages, did not contain 'carotene' or 'xanthophyll', vitamin A_1 or A_2 or 7-dehydrocholesterol in detectable amounts.

2. Astaxanthin was the sole detectable carotenoid pigment. In the course of development, most of it is transferred to the embryo and esterified.

3. More than 40 % of the neutral fat is metabolized.

4. Marked synthesis of cholesterol occurs in the period immediately before and after hatching and there is a concomitant and equivalent disappearance of phospholipin.

We are indebted to the Ministry of Food and the Medical Research Council for financial assistance during the period in which the work was carried out.

REFERENCES

Anno, K. (1940). *J. agric. chem. Soc. Japan* (Bull.), **16**, 181. Cited in *Chem. Zbl.* (1941), **1**, 2814.
Channon, H. J. (1925). *Biochem. J.* **19**, 424.
Dam, H. (1928). *Biochem. Z.* **194**, 188.
Daniel, R. J. (1947). *J. exp. Biol.* **24**, 123.
Halpern, G. R. (1945). *Nature, Lond.*, **155**, 110.
Hanes, F. M. (1912). *J. exp. Med.* **16**, 512.
Hayes, F. R. (1930). *Biochem. J.* **24**, 3, 735.
Hayes, F. R. & Hollett, A. (1940). *Canad. J. Res.* **182**, 53.
Hayes, F. R. & Hollett, A. (1946). *Canad. J. Res.* **24** D, 39
Hayes, F. R. & Ross, D. M. (1937). *Proc. roy. Soc.* B, **122**, 358.
Hevesy, B. V., Levi, H. & Rebbe, O. (1938). *Biochem. J.* **32**, 2147.
Johnstone, J. A. L. & Hayes, F. R. (1938–42). *Proc. N.S. Inst. Sci.* **20**, 31.
Lovern, J. A. (1934). *Biochem. J.* **28**, 1955.
Lovern, J. A. (1936). *Biochem. J.* **30**, 20.
Miescher (1866). Cited by Hayes, F. R. & Ross, D. M. (1937).
Morton, R. A. & Rosen, D. G. (1949). *Biochem. J.* **45**, 612.
Mueller, J. H. (1915). *J. biol. Med.* **21**, 23.
Needham, J. (1931). *Chemical Embryology*, Vol. II, p. 1221. Cambridge University Press.
Riddle, O. (1916). *Amer. J. Physiol.* **41**, 409.
Rittenberg, D. & Schoenheimer, R. (1937). *J. Biol. Chem.* **121**, 235.
Roffo, A. H. & Azaretti, I. (1926). *Bull. Int. Med. Exptl.* **2**, 629.
Steven, D. M. (1947). *Nature, Lond.*, **160**, 507.
Thannhauser, S. J. & Schaber, H. (1923). *Hoppe-Seyl. Z.* **127**, 278.
Yamaguchi, M. (1924). *Trans. Jap. path. Soc.* **14**, 227. Cited by Needham (1931).

Some Observations on the Zinc in Carbonic Anhydrase

By R. TUPPER, R. W. E. WATTS and A. WORMALL
Departments of Biochemistry and Physiology, Medical College of St Bartholomew's Hospital, London

(*Received* 18 *June* 1951)

Keilin & Mann (1939, 1940) showed that carbonic anhydrase is a zinc protein and this has been confirmed by many authors (Hove, Elvehjem & Hart, 1940; Scott & Fisher, 1942, 1944; see also the reviews by Vallee & Altschule, 1949*a*, *b*). The nature of the linkage between the metal and the protein is unknown (cf. the review by Roughton & Clark, 1951), but the zinc is undoubtedly firmly bound and is not removed by dialysis, even when the enzyme has become inactivated by long standing (Keilin & Mann, 1940).

Red blood cells and leucocytes can readily be labelled *in vitro* with ^{65}Zn by adding a solution of a ^{65}Zn-containing zinc-glycine complex to the cell suspension and keeping the mixture at 37° and pH 7·4 for 2 hr. (Tupper, Watts & Wormall, 1951). A further study of the distribution of the ^{65}Zn in these labelled red cells has shown that, although about 94 % of the ^{65}Zn which has become fairly firmly attached to the cells is associated with the cell proteins, little or none is present in the carbonic anhydrase. In view of these results we decided to find out, using ^{65}Zn, whether *in vitro* exchange of zinc between zinc salts and carbonic anhydrase is possible if the mixtures are kept for a long time. The results of these and some closely related investigations are described in this paper.

EXPERIMENTAL

Materials

Carbonic anhydrase. The enzyme was prepared from washed ox erythrocytes by 'method II' of Keilin & Mann (1940). Two different batches (*A* and *B*) were used, corresponding, respectively, to solution (*a*) and the most highly purified preparation described by these authors. *A* and *B* contained, respectively, 383 and 158 μg. of protein, based on 14·95 % N in the enzyme (Keilin & Mann, 1940), and 120 and 126 enzyme units/ml. Assuming that the purer batch (*B*) contained no other nitrogenous compound, this enzyme preparation contained 0·33 % of Zn. Batch *A* was used for experiments 1 and 2 (Table 1) and *B* for Exp. 3.

Zinc-glycine complex. Glycine (4·0 mg.) was added to a ^{65}Zn-containing $ZnCl_2$ solution (0·5 ml. containing 105 μg. Zn and giving 8225 counts/min. with our counting system), and 0·01 N-NaOH was added dropwise until the pH was about 7·4. The solution was diluted to 1·0 ml. with glass-distilled water and used for Exps. 1 and 2. For Exp. 3 double the above amounts of glycine and $ZnCl_2$ solution were used and the mixture was diluted to 4·5 ml.

Ammonium sulphate. Since the purest available (Analar) samples of $(NH_4)_2SO_4$ contain significant amounts of Zn, it was necessary to remove this metal before the salt was used in these 'exchange' investigations. A saturated aqueous solution of the salt was made alkaline to phenol red with NH_3, and the solution was shaken with a solution of dithizone (diphenylthiocarbazone) in CCl_4 until no more Zn

could be extracted. The dithizone remaining in the aqueous solution was then extracted with CCl_4, and the $(NH_4)_2SO_4$ solution evaporated to dryness in an evaporating basin which had previously been washed, successively, with conc. HNO_3, glass-distilled water, a solution of dithizone in CCl_4 and finally CCl_4. The Zn-free $(NH_4)_2SO_4$ obtained was used for the final precipitation in the preparation of the enzyme, and also for precipitation of the enzyme from the Zn-glycine-enzyme mixtures.

Distilled water. For dialysis and for the preparation of all the solutions used in this investigation, glass-distilled water was used. This was found to contain not more than 0·44 μg. in contrast to a value of 32 μg. Zn/l. for the water from a 'Manesty' still.

Methods

Carbonic anhydrase activity. The enzyme activity was determined by the method of Meldrum & Roughton (1934) and expressed in terms of the carbonic anhydrase unit defined by these authors. The boat used was essentially that described by Brinkmann, Margaria & Roughton (1933) and Meldrum & Roughton (1934).

Zinc determinations. The dithizone method as modified by Gettler & Bastian (1947) was used. The material was digested with acid and the digest was brought to pH 8·5–9·0 with NH_3 as described by Banks, Tupper & Wormall (1950). The zinc was then extracted by repeated shaking with dithizone solution (40 mg./l. of CCl_4), and the amount of Zn present calculated by comparing the amount of dithizone solution required with that needed to give complete extraction of the metal from standard $ZnSO_4$ solutions under comparable conditions. Blank determinations were carried out and allowance was made for the small amount of Zn in the digestion mixture and other reagents used.

Radioactivity determinations. All radioactivity determinations were made with the liquid counter described by Veall (1948); solutions with a relatively low salt content, e.g. the purified carbonic anhydrase preparations and solutions of zinc-glycine complexes, were 'counted' without further treatment. Solutions containing appreciable amounts of $(NH_4)_2SO_4$, e.g. undialysed solutions of $(NH_4)_2SO_4$-precipitated carbonic anhydrase, were digested with acid and the ^{65}Zn extracted and measured as described by Banks *et al.* (1950).

RESULTS

Attempted exchange of zinc between carbonic anhydrase and a zinc-glycine complex

A solution of ^{65}Zn-containing zinc-glycine complex was added to solutions of carbonic anhydrase and the mixtures were kept in the refrigerator for varying lengths of time, after which the enzyme was precipitated by ammonium sulphate. The enzyme activity and the ^{65}Zn content of the precipitates were determined to find out whether any exchange of ^{65}Zn had occurred. A typical experiment (Exp. 2, Table 1) was carried out as follows.

The carbonic anhydrase solution (15 ml. of batch *A*) was mixed with a solution of labelled zinc-glycine complex (0·5 ml. containing 52·5 μg. of Zn) and the mixture kept at 0–4°. Radioactivity and enzyme activity determinations were made at the start and after 8 days. At the end of this storage period a sample (10 ml.) of the mixture was saturated with $(NH_4)_2SO_4$, and the precipitated carbonic anhydrase was filtered off, washed with five 2 ml. portions of saturated $(NH_4)_2SO_4$ solution and dissolved in glass-distilled water (10 ml.). Radioactivity and carbonic anhydrase determinations were made on this solution, and, as a check, the ^{65}Zn contents of the filtrate and washings were determined.

In another similar experiment the mixture was kept for 32 days at 0–4°, and then divided into two equal portions for precipitation of the enzyme by $(NH_4)_2SO_4$. In this experiment the yield of carbonic anhydrase in the precipitate was unexpectedly low, but sufficient was obtained for the purposes of this investigation.

The results of these experiments (Table 1) show that no significant amount of ^{65}Zn entered the carbonic anhydrase molecule even when the mixtures were kept for 32 days. Calculations based on the total zinc contents of the enzyme and the zinc-glycine complex, and the ^{65}Zn content of the latter, indicated that considerable amounts of ^{65}Zn would have been present in the precipitated

Table 1. *Attempt to obtain* in vitro *exchange of* ^{65}Zn *between a* ^{65}Zn*-containing zinc-glycine complex and carbonic anhydrase*

(Experimental details are given on p. 430.)

		Mixture				$(NH_4)_2SO_4$-precipitate		
		Total zinc					^{65}Zn	
Exp. no.	Period available for exchange (days)	In the mixture (μg.)	In the carbonic anhydrase (μg.)	^{65}Zn (counts/min.)	Carbonic* anhydrase (units)	Carbonic anhydrase (units)	Found (counts/min.)	Predicted† (counts/min.)
1	1	73	22	4114	1710	780	0‡	565
2	8	49	15	2732	1360	690	0‡	425
3	32	47	6·2	2555	756	(*a*) 130	0‡	58
						(*b*) 142	0‡	63

(*a*) and (*b*) refer to separate precipitations of the enzyme.

* Determined at the end of the period of storage.

† Calculated on the assumption that there would be free exchange between the zinc of the zinc-glycine complex and that in the carbonic anhydrase.

‡ Counts within the variation of the background (i.e. less than 3).

enzyme if there had been free exchange of zinc between the enzyme and the complex (see last column of Table 1).

'Availability' of the ^{65}Zn in the zinc-glycine complex

In these experiments with carbonic anhydrase, and in other investigations on the labelling of blood cells with ^{65}Zn, we have kept the mixtures at about pH 7·4. The direct addition of ordinary inorganic zinc salts to the enzyme solution or cell suspension was thus precluded, since the metal is readily precipitated at this pH even from very dilute solutions. We have therefore added sufficient glycine to the zinc chloride solution to prevent the formation of a precipitate when alkali was added to bring the mixture to pH 7·4.

It might be contended that the zinc in this zinc-glycine complex may be relatively firmly bound and thus unable to undergo exchange reactions. To test this possibility we have studied the availability of the zinc in the complex, and have found that the metal is readily freed from its combination with glycine. For example, the addition of phosphate (as 0·5 M-phosphate buffer of pH 7·4) to a solution of the zinc-glycine complex at pH 7·4 leads to almost complete precipitation of the zinc as phosphate; all the glycine remains in the supernatant solution, and no significant change occurs in the pH of the solution. In other tests we have found that the zinc of the zinc-glycine complex is readily extracted at pH 7·4 when the solution, or a mixture of blood plasma and zinc-glycine solution, is shaken with a solution of dithizone in carbon tetrachloride. Thus the ^{65}Zn of the zinc-glycine complexes used here was freely available for exchange with other zinc compounds in the systems studied. Furthermore, it is known that the stable complexes of zinc and other heavy metals with amino-acids 'are in equilibrium with a (usually exceedingly minute) proportion of inorganic ions from which they are formed' (Albert, 1950).

Zinc in the carbonic anhydrase of ^{65}Zn-labelled red blood cells

Most of the ^{65}Zn which enters or becomes attached to red blood cells when they are exposed to a solution of ^{65}Zn-containing zinc-glycine complex and then washed with 0·9 % NaCl (w/v) is relatively firmly bound (Tupper *et al.* 1951), and experiments were therefore carried out to determine the proportion of the bound ^{65}Zn which is associated with the carbonic anhydrase in these labelled erythrocytes.

In a preliminary experiment to determine how much of the ^{65}Zn is attached to the total protein of red cells, washed rabbit erythrocytes (5 ml.) were treated with a ^{65}Zn-containing zinc-glycine solution at pH 7·4 and kept for 2 hr. at 37°. The cells were then washed thrice with 0·9 % NaCl (5 ml. each time) and then lysed by adding distilled water (25 ml.). The mixture was centrifuged and the deposited stroma was washed with 0·9 % NaCl until the washings were colourless. (This washed stroma contained less than 1 % of the ^{65}Zn of the laked erythrocyte solution.) The stroma washings (20 counts/min.) were added to the haemoglobin solution and the mixture (4310 counts/min.) was saturated with $(NH_4)_2SO_4$. The mixture was kept overnight in a refrigerator, and then warmed gently in a water bath (to aid flocculation of the precipitate) and filtered. The precipitate was washed well with saturated $(NH_4)_2SO_4$ solution, and radioactivity determinations were made on the filtrate, the washed precipitate and the washings.

The results showed that the washed precipitated proteins contained about 94 % and the filtrate only about 1 % of the total ^{65}Zn of the labelled red cells. It is of interest that the insoluble protein (stroma) of the lysed red cells contained only a minute proportion of the total ^{65}Zn, since this apparently excludes the possibility that the radioactivity of the labelled red cells is due to adhering precipitated inorganic zinc salts.

In view of these results, other experiments were carried out to determine what fraction of the cell-bound ^{65}Zn is attached to the carbonic anhydrase of the *in vitro* labelled erythrocytes.

Table 2. ^{65}Zn *content of the carbonic anhydrase of ox erythrocytes labelled with* ^{65}Zn

(The values given below are those for 20 ml. of labelled ox erythrocytes, or fractions obtained from this amount. For further details see p. 431.)

	Carbonic anhydrase (units)	^{65}Zn content (counts/min.)
Lysed erythrocytes	28,800	20,300
Separated crude carbonic anhydrase*	17 900	40
Denatured protein	—	21,500

* Separated by adding ethanol-$CHCl_3$ to the lysed erythrocytes.

Fresh defibrinated ox erythrocytes (20 ml.) were washed well with 0·9 % NaCl and then treated with 4·5 ml. of a zinc-glycine solution (pH 7·4 and with NaCl added to make the solution isotonic with ox erythrocytes; total counts, 26,900/min.). The mixture was kept at 37° for 2 hr. and then centrifuged. The labelled cells were washed thrice with 0·9 % NaCl (20 ml.) and lysed by adding distilled water (10 ml.). ^{65}Zn and carbonic anhydrase determinations were made on samples of this solution, and the remainder (26 ml.) was cooled to 0° and treated with 17 ml. of an ice-cold ethanol-$CHCl_3$ mixture (1 vol.: 1 vol.). The mixture was stirred well, kept at 0° for 15 min. and then centrifuged for 30 min. at 0°. The supernatant solution was dialysed against running water for 24 hr. and the radioactivity and carbonic anhydrase activity of the product ('$CHCl_3$-enzyme') were determined. The precipitate from the water-ethanol-$CHCl_3$ mixture ('denatured protein') was dried at 120°, boiled down to a small volume after adding conc. HNO_3 (30 ml.) and the solution filtered through glass wool and then diluted to 50 ml.; a radioactivity determination was made on this solution.

The results (Table 2) showed that the separated carbonic anhydrase fraction (representing about two-thirds of the original enzyme of the lysed red cells) contained less than 0·25 % of the ^{65}Zn present in the labelled cells. Essentially the whole of the ^{65}Zn was present in the 'denatured protein' fraction. In view of the very low ^{65}Zn content of the relatively crude '$CHCl_3$-enzyme' preparation, it was decided that it was unnecessary, for the purposes of this experiment, to continue with the purification of the enzyme, since the small amount of purified enzyme which would have been obtained, would almost certainly have contained a negligible amount of ^{65}Zn.

DISCUSSION

The results of these investigations with ^{65}Zn are in complete agreement with the observations of Keilin & Mann (1940) that the zinc in carbonic anhydrase is firmly bound to the protein. The absence of exchange *in vitro* of zinc between the enzyme and solutions containing available ^{65}Zn emphasizes the similarity between the stability of this metal-protein complex and that of certain other metallo-proteins. For example, Ruben, Kamen, Allen & Nahinsky (1942) have found that there is no significant exchange of iron between methaemoglobin ('ferrihaemoglobin' and 'ferrihaem') and radioactive ferric salts when kept in contact for 2 months, and Ruben, Frenkel & Kamen (1942) observed no exchange *in vitro* of magnesium between chlorophylls *a* and *b* and magnesium ions.

SUMMARY

1. Carbonic anhydrase kept as long as 32 days at 0° and pH 7·4 in a solution of ^{65}Zn-containing zinc salts (plus glycine to prevent precipitation of the zinc) takes up no significant amount of ^{65}Zn.

2. When red blood cells are labelled *in vitro* with ^{65}Zn little or none of the zinc which enters the red cell is firmly attached to the carbonic anhydrase.

3. The inability of carbonic anhydrase to exchange its zinc with zinc ions and simple zinc compounds supports the view of Keilin & Mann (1939, 1940) that the metal which is an integral part of the enzyme molecule is firmly bound to the protein.

We are pleased to express our indebtedness to the British Empire Cancer Campaign for grants which have covered the main expenses of this investigation. The ^{65}Zn was separated from copper or phosphor bronze strips irradiated in the Birmingham and Liverpool cyclotrons and we are very grateful to Prof. P. B. Moon, F.R.S., Prof. H. W. B. Skinner, F.R.S., and their colleagues for having supplied this material to us.

REFERENCES

Albert, A. (1950). *Biochem. J.* **47**, 531.
Banks, T. E., Tupper, R. L. F. & Wormall, A. (1950). *Biochem. J.* **47**, 466.
Brinkmann, R., Margaria, R. & Roughton, F. J. W. (1933). *Philos. Trans.* A, **232**, 65.
Gettler, A. O. & Bastian, R. (1947). *Amer. J. clin. Path.* **17**, 244.
Hove, E., Elvehjem, C. A. & Hart, E. B. (1940). *J. biol. Chem.* **136**, 425.
Keilin, D. & Mann, T. (1939). *Nature, Lond.*, **144**, 442.
Keilin, D. & Mann, T. (1940). *Biochem. J.* **34**, 1163.
Meldrum, N. U. & Roughton, F. J. W. (1934). *J. Physiol.* **80**, 113.
Roughton, F. J. W. & Clark, A. M. (1951). *The Enzymes. Chemistry and Mechanism of Action*, vol. 1, pt. 2, edited by Sumner, J. B. & Myrbäck, K., chap. 43. New York: Academic Press.
Ruben, S., Frenkel, A. W. & Kamen, M. D. (1942). *J. phys. Chem.* **46**, 710.
Ruben, S., Kamen, M. D., Allen, M. B. & Nahinsky, P. (1942). *J. Amer. chem. Soc.* **64**, 2297.
Scott, D. A. & Fisher, A. M. (1942). *J. biol. Chem.* **144**, 371.
Scott, D. A. & Fisher, A. M. (1944). *Nature, Lond.*, **153**, 711.
Tupper, R., Watts, R. W. E. & Wormall, A. (1951). *Biochem. J.* **48**, xxxvii.
Vallee, B. L. & Altschule, M. D. (1949*a*). *Physiol. Rev.* **29**, 370.
Vallee, B. L. & Altschule, M. D. (1949*b*). *Blood*, **4**, 398.
Veall, N. (1948). *Brit. J. Radiol.* **21**, 347.